A STUDY OF IMMUNE RESPONSES TO *RAS* TRANSFORMED TUMOUR CELLS EXPRESSING WELL-DEFINED ANTIGENS

Jennifer Linda Johnson

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Department of Biological Sciences

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Declaration

I declare that all the work in this thesis, except where specifically stated, was original research performed by myself under the supervision of Dr. Alan Morris at the Department of Biological Sciences, University of Warwick. None of this work has previously been submitted for any degree. All sources of information have been acknowledged by means of reference.

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Abbreviations

AMP	ampicillin
AMPS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
DNA	deoxyribonucleicacid
DTT	dithiothreitol
DW	distilled water
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
FACS	flourescence activated cell sorter
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
kb	kilo bases
kbp	kilobase pairs
kD	kilo daltons
Ki	Kiraten
LB	Luria-Bertani medium
MLV	murine leukaemia virus
Mo	Moloney
mRNA	messenger ribonucleic acid
MSV	murine sarcoma virus
m. wt.	molecular weight
NEO^R	neomycin resistance
OD	optical density
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
Tc	cytotoxic T lymphocyte
Th	helper T lymphocyte
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol

Summary

In this work tumour cells expressing well-defined antigens were generated to study the importance of interferon (IFN) modulation of T cell mediated immune responses to foreign antigens. The antigens used were those of a murine leukaemia virus (MLV). DNA clones of this virus were manipulated using standard molecular biology techniques to generate replication-defective viruses expressing individual MLV antigens (*env* or *gag*) when transfected into tumour cells. The tumour cell line used in this study (C3H201) was produced in our laboratory by the transformation of murine fibroblasts (C3H10T½) with the Kirsten murine sarcoma virus (MSV) which expresses the *ras* oncogene.

Initially cells transfected with the MLV genes were selected by neomycin resistance and preliminary investigations using these cells suggested that *gag* is the main T-cell target in this system; the *gag* expressing cells were more susceptible to MSV/MLV-specific Tc lysis and were less tumourigenic than the *env* transfected cell lines. However it was found that the cells expressing the neomycin resistance (neo^R) gene alone also elicited an immune response (increased susceptibility to Tc lysis and less tumourigenic than the untransfected C3H201 cells) thus it was suggested that this selection system was not suitable for cells to be used in immunological studies and an alternative selection system was needed.

To overcome the problems observed with neomycin resistance a novel selection system using the major histocompatibility (MHC) antigen D^b was developed. The H-2D^b gene was transfected into the H-2D^b negative cell line C3H201 ($H\text{-}2^a$) with the DNA of interest and then the cells expressing the D^b antigen were sorted on the FACS following IFN-γ treatment and indirect immunofluorescence staining. Experiments to determine the immunological importance of the antigen of interest were then performed in the F1 progeny from a cross between C3H/He ($H\text{-}2^a$) and C57BL6 ($H\text{-}2^b$) mice which recognise the endogenous and acquired MHC antigens of the transfected cell line as self. This selection system thus enabled the examination of the immune response to the proteins of interest without actually interfering with the response they elicit.

This system was used to produce *gag* expressing cells and also a cell line that expresses all the MLV proteins without producing the virus itself (designed to facilitate an examination of the effect of virus replication and subsequent infection on the response to MLV antigens by comparison with a MLV expressing cell line). These cell lines were found to be susceptible to lysis by Mo-MSV/MLV-specific Tc and this lysis was increased by IFN-γ treatment (cells transfected with D^b alone, DC3H201, showed no such lysis). It is now known that Tc usually recognise antigen in association with class I MHC antigen. Furthermore IFN-γ treatment of C3H201 cells has been shown to increases class I MHC antigen expression on these cells. Thus it is proposed that the increased susceptibility to lysis observed for these IFN-γ treated cells is due to increased class I MHC antigen expression. These cells also show reduced tumourigenicity in mice in comparison with DC3H201 and C3H201 thus confirming the recognition of *gag* by the immune system (in the case of DN-gag1 and DN-gag2) and furthermore the correlation between the tumourigenicity and susceptibility to Tc lysis of all these cells supports the importance of the Tc element in the immune response to these tumour cells.

1.1 Introduction

The main aim of this work was to generate tumour cell lines expressing well-defined antigens which could then be used to study the importance of interferon (IFN) modulation of T cell mediated immune responses to these antigens. The antigens used in this study were the *gag* and *env* polypeptides expressed by the Kirsten (Ki) and Moloney (Mo) murine leukaemia virus (MLV). These viruses are classified as replication-competent but transformation-defective as they lack any oncogene and thus do not transform tissue culture cells. DNA clones of these viruses were manipulated using standard molecular biology techniques to generate replication-defective viruses expressing individual MLV antigens (*gag* or *env*) when transfected into tumour cells. The tumour cell line used in this study (C3H201) was produced in our laboratory by the transformation of a murine fibroblast cell line (C3H10T½) with Ki murine sarcoma virus (MSV) which expresses the *ras* oncogene. This transformation-competent virus, similar to Mo MSV, is replication-defective as it lacks the genes required for replication however the proteins encoded by the replication genes can be supplied in *trans* by the closely related replication-competent virus. C3H10T½ derived cells were used in this study as they are reported to be free of 'overt' endogenous transforming murine leukaemia or sarcoma viruses (see ATCC catalogue). Moreover, the C3H/He inbred strain of mice (from which the C3H10T½ cells derive) is currently being used by our laboratory for other immunological investigations, making it readily available. It is hoped this study will aid these investigations.

The inoculation of mice with cells infected with MSV/MLV expressing MLV antigens results in the induction of tumours. It has been shown that at certain cell concentrations these tumours regress. This is due to an immune

response to the MLV antigens, as tumours of cells expressing MSV alone show no such regression at the same cell concentration. By using the tumour cells expressing the individual MLV antigens my intention was to examine the relative importance of *gag* and *env* in tumour regression. Furthermore, a molecular clone of the MLV genome lacking the ψ packaging signal was to be used to examine the response to the MLV antigens together, though without the complications associated with the presence of infectious virus.

Evidence presented in this study indicates that neomycin resistance is not a suitable selection system for cells to be used in immunological studies. It is shown that tumour cells transfected with just the neomycin resistance (neo^R) gene have immunological properties different to those exhibited by the cells they were derived from (increased susceptibility to Tc lysis and reduced tumourigenicity). It is proposed that that the bacterial protein itself acts as a target to the immune system. A further aim of this project therefore was to develop a selection system that does not itself alter the immunological properties of the transfected cells. The system developed uses the major histocompatibility antigen D^b and is described in detail later in this chapter.

In the following literature review areas relevant to this study are examined. As the main aim of my work was to develop tumour cells expressing well-defined antigens for the study of immune responses to these tumour cells (in particular T cells) I will first discuss briefly the nature of the T cell response playing particular attention to the role of MHC antigens (section 1.2). The following section reviews the evidence for an immune response to both human and murine tumours and briefly examines the mechanisms for evading immune surveillance. One of these mechanisms, modulation of MHC antigens is then reviewed in some detail in section 1.4.

Section 1.5 briefly discusses the effector mechanisms involved in immune responses to tumours. Section 1.6 introduces the MSV/MLV system and section 1.7 then examines the substantial evidence for an immune response to tumours within this system. Finally in section 1.8 the strategy for introducing the defined antigens into the cells is described, including some general background on the use of retroviral vectors and selection systems.

1.2 The T cell response

The T cell response can essentially be divided into two types; helper cells (Th) and cytotoxic T cells (Tc). Th cells were originally defined in functional assays as being able to induce B lymphocytes to secrete antibody. The binding of Th cells to B cells causes the B cells to synthesize receptors for the B cell growth factor interleukin-4 (IL-4) (Harlow and Lane, 1988) which is secreted by activated Th cells (Killar *et al.*, 1987). B cells that have bound IL-4 start to proliferate and synthesize cell-surface receptors for the B cell differentiation factor IL-5 which is also secreted by by activated Th cells. The binding of IL-5 to proliferating B cells induces the cells to differentiate into plasma cells (which secrete antibody) and memory cells (Harlow and Lane, 1988). Th cells also constitutively express receptors for IL-1 which is produced by a variety of cells including macrophages, monocytes, endothelial cells, dendritic cells and epithelial cells (Male *et al.*, 1987). On recognition of foreign antigen on an antigen presenting cell Th cells bind IL-1 and are thus induced to express receptors for IL-2 and and to release IL-2 (T cell growth factor), and IFN-γ (Male *et al.*, 1987). IL-2 stimulates the proliferation of Tc and Th cells which have undergone recognition of antigen and thus express IL-2 receptors (Male *et al.*, 1987).

Tc are a subpopulation of small lymphocytes which are important in the killing of virally infected cells, tumour cells (as discussed in the next section), and cells in both allogeneic and xenogeneic transplants. The interaction of Tc with these different target cells has been divided into three distinct phases; recognition and adhesion, programming the target for lysis, and then lysis which is independent of the continued presence of the Tc (Yannelli *et al.*, 1986). Although much is now known about the

recognition phase (see below), the mechanism of lysis is still unclear. After recognition of the antigen the Tc are induced to express receptors for IL-2 and proliferate in response to IL-2 (Kaplan *et al.*, 1984). They also release IL-2 and IFN-γ on recognition of the antigen on a target cell (Morris *et al.*, 1982; Taylor *et al.*, 1985). The IL-2 may then induce further Tc proliferation resulting in an antigen specific T cell response. The release of IFN-γ has many immunomodulatory effects and one of these effects is discussed below.

It has long been shown that the majority of Tc belong to a subset of T cells, $Lyt2^+$ in mice and $CD8^+$ in humans, which recognise antigen in association with class I molecules of the MHC, whereas helper T cells are $L3T4^+$ ($CD4$ in humans) and class II restricted (Cantor and Boyse, 1975). However, it is now known that a minority of Tc are also $L3T4^+$ and recognise antigens in association with class II MHC molecules, thus the correlation between MHC restriction of a T lymphocyte antigen receptor and the cell's functional activity or surface phenotype is not complete, (Hauer *et al.*, 1982; Spits *et al.*, 1983; Swain *et al.*, 1983; Braciale *et al.*, 1987).

MHC restriction

Prior to discussing MHC restiction, a brief introduction to the MHC will be given. The MHC is a highly polymorphic region of the genome whose products play a major role in the immune response as already mentioned earlier. It is known as the MHC for historical reasons since the molecules of this complex were first recognised as classical transplantation antigens on the surface of cells. The MHC in humans, known as the human leukocyte antigen (HLA) system, is coded for by a genetic region located on the short arm of chromosome 6. In the murine system the MHC has been mapped to a genetic region (termed H-2) on chromosome 17 (McConnell *et al.*, 1984). The

haplotypes of the mice used in this study are H-2^{k} (C3H/He), H-2^{b} (C57 Bl/6) and H-2^{bk} (F1 hybrid). Three classes of molecules, denoted I, II and III, have been identified in the MHC of both mouse and humans. At least three separate class I loci (termed H-2K, -D and -L in the mouse and HLA-A, -B and -C in humans) encoding classical transplantation antigens have been demonstrated. Other class I genes in the mouse map at the right of the MHC in regions known as Qa-2,3 and Tla (Hale *et al.* 1987). Class II genes, encoded in the I-A and I-E regions of the mouse MHC and the HLA-D region of humans, are identical to the immune response (Ir) genes known to control murine responses to different antigens (see later). The class III genes which encode several components of the complement system will not be discussed further here. The major feature of the MHC is its high level of polymorphism. For instance, more than 50 different alleles have been demonstrated at both the H-2K and H-2D class I loci of mice. Class I molecules are generally more polymorphic than class II molecules, although the Qa-2,3 and Tla region class I genes exhibit less polymorphism.

The class I MHC antigens are comprised of a glycosylated polypeptide chain of 45 KD (heavy chain) in close non-covalent association with β_2-microglobulin, a 12 KD polypetide (Law *et al.*, 1986). β_2-microglobulin is encoded outside the MHC on human chromosome 15 (Goodfellow *et al.*, 1975) and on mouse chromosome 2 and appears to stabilise the class I MHC molecule (Hale *et al.*, 1987). Amino acid sequence analyses of human and murine class I molecules have demonstrated that the heavy chain is divided into five distinct regions: three extracellular domains of about 90 amino acids designated α_1, α_2 and α_3, a transmembrane region of about 25 hydrophobic amino acids, and a cytoplasmic domain which consists of about 30 hydrophilic amino acids. Intrachain disulphide bonds in the α_1, α_2 and β_2-microglobulin domains stabilise the structure of the molecule. As

discussed later Bjorkman *et al.* (1987a, 1987b) determined the three-dimensional structure of the human class I MHC HLA-A2 antigen by x-ray crystallography and confirmed the presence of the α_1, α_2, α_3 and β_2-microglobulin domains. Class I MHC antigens can be detected on the surface of virtually all nucleated cells (except hepatocytes and cells of the central nervous system, exocrine pancreas and cornea-endothelium and as discussed elsewhere are involved in the presentation of antigens to Tc.

The class II MHC antigens are heterodimers comprising heavy (α) and light (β) glycoprotein chains. The α chains have molecular weights of 30 to 34 KD and the β chains range from 26-29 KD, depending on the alleles involved (Male *et al.*, 1987). On the basis of amino acid sequence, each chain has been shown to consist of four domains; two extracellular domains of about 90 amino acids designated α_1 and α_2 or β_1 and β_2, a transmembrane region of about 30 amino acids, and a short cytoplasmic domain of between 10 and 15 hydrophilic amino acids (Male *et al.*, 1987). Only a few cell types constitutively express class II MHC antigens (e.g. B-cells and dendritic cells; Knight *et al.*, 1987; Male *et al.*, 1987) although a number of other cell types can be induced to express class II antigens by IFN-γ treatment (e.g. murine fibroblasts, astrocytes and macrophages; Maudsley and Morris, 1989b; Tomkins *et al.*, 1988; Wong *et al.*, 1984a).

The phenomenon of MHC restriction was first described and named by Zinkernagel and Doherty (1974) (reviewed by Schwartz, 1985). They showed that Tc from mice infected with lymphocytic choriomeningitis virus (LCMV) could kill LCMV-infected target cells only if the targets possessed the same set of genetic determinants located at the H-2 complex (i.e. were of the same haplotype). Further work with H-2 recombinant mice found that this

restriction mapped to the K and D regions of the H-2 (Zinkernagel and Doherty, 1975; Blanden *et al.*, 1975; for review see Zinkernagel and Doherty, 1979). McMichael (1978) showed that these observations could be extended to the human system as it was shown that influenza-specific Tc only killed infected target cells that shared identical HLA-A and HLA-B locus antigens.

Many groups have shown that Tc recognition of a target cell is directly related to the level of MHC antigen expression on the cell. The property of the enzyme papain to remove MHC antigens from the surface of target cells was utilised by Kuppers *et al.* (1981) to examine the relationship between levels of MHC antigen on the target cell and lysis by Tc. Murine target cells treated with papain, prior to haptenation (trinitrophenyl-modified (TNP)) were found to be less susceptible to lysis by TNP-specific Tc than cells not treated with papain. Additionally, the decrease in susceptibility followed the same dose and time kinetics as the reduction of MHC antigens expressed on the cell surface as assessed by serological assay and susceptibility to lysis by alloreactive Tc.

Th cells are activated by antigens presented by accessory cells which display class II MHC antigens and again the Th cells are only activated if the presenting cells are of the same haplotype. Much of the work to investigate the relationship between expression of class II antigens and ability to present antigen had been studied using macrophages. Although cultured macrophages do not constitutively express class II MHC antigens they can be induced to do so with IFN-γ (Beller, 1984) as discussed in some detail below. Beller and Ho (1982) found that the ability to present antigen to Th cells was directly correlated to the expression of class II MHC antigens by these macrophages.

Modulation of MHC antigen expression by interferon

There are three main classes of murine and human interferon (IFN) which have been identified to date; α, β and γ which were classified on the basis of antigenicity using polyclonal antisera (Committee on Interferon Nomenclature, 1980). IFNs α and β, which are also called type I or viral IFNs are produced by most cell types when infected with virus (De Maeyer, 1984). IFN-αβ will be used when referring to both IFN-α and IFN-β in this thesis. IFN-γ, which is also called type II or immune IFN, is produced mainly by T-lymphocytes subsequent to stimulation by specific antigen or mitogen (Klein *et al.*, 1982), although NK cells also produce this IFN.

Since the original work by Lindahl *et al.* (1973) and Sonnenfeld *et al.* (1981) for IFN-αβ and IFN-γ respectively, it has been shown by many groups that both human and murine IFNs modulate MHC antigen expression on a wide variety of cells cultured *in vitro*. Generally, IFNs-αβ have been shown to induce only class I MHC antigen expression whereas IFN-γ has been shown to induce not only class I MHC antigen expression but also class II on certain cell types. For example, IFNs-αβ have been shown to induce class I, but not class II, MHC antigen expression on human melanoma cells (Imai *et al.*, 1981), murine astrocytes (Wong *et al.*, 1984b), human keratinocytes (Wiedarwieser *et al.*, 1988) and murine fibroblasts (Morris and Tomkins, 1989). Alternatively, IFN-γ has been shown to induce both class I and class II MHC antigen expression on murine fibroblasts and glial cells (Maudsley and Morris, 1988; Morris and Tomkins, 1989), murine B cells, macrophages and mast cells (Wong *et al.*, 1984a), human keratinocytes (Wiedarwieser *et al.*, 1988), murine astrocytes (Wong *et al.*, 1985) and human melanoma cells (Rosa *et al.*, 1986). It has been shown that the different IFN classes interact with each other in the induction of MHC antigen expression. IFN-α and IFN-β have an additive effect with IFN-γ in the induction of class I

MHC antigen expression on murine fibroblasts and glial cells (Morris and Tomkins, 1989) and similarly human endothelial cells (Lapierre *et al.*, 1988). In contrast, IFN-α and IFN-β have been shown to inhibit the ability of IFN-γ to induce class II MHC antigen expression on murine fibroblasts and glial cells (Morris and Tomkins, 1989), human endothelial cells (Lapierre *et al.*, 1988; Manyak *et al.*, 1988) and murine macrophages (Fertsch *et al.*, 1987; Kitaura *et al.*, 1988).

Considering that the susceptibility of target cells to Tc lysis is directly related to the levels of class I MHC antigen expressed on the surface of the cell, it is not surprising that the IFNs (α, β, γ), which augment the expression of class I MHC antigen, also increase the susceptibility of target cells to lysis by Tc. For example, Morris *et al.* (1987) found that IFN-αβ treatment of Semliki Forest virus (SFV) infected primary brain cells resulted in an augmentation of lysis of these cells by SFV-specific Tc. All three classes of interferon have been shown to increase the susceptibility of certain cell types to alloreactive Tc lysis. For example, murine fibroblasts and lymphoblastoid cells (Blackman and Morris, 1985), murine fibroblasts and bone marrow cells (Bukowski and Welsh, 1986) and rat mammary carcinoma cells (Yeoman and Robins, 1988). Similarly, as discussed above activation of Th cells is directly related to the level of class II MHC antigen expression by the presenting cell and therefore IFN-γ increases Th activation.

It is now known that viruses may also modulate host cell MHC antigen expression, for example, measles virus has been shown to induce class I and class II MHC antigen expression on their host cell (Massa *et al.*, 1986; Massa *et al.*, 1987a). This is examined in detail in section 1.6 and therefore will be discussed no further here.

The T cell antigen receptor

To date there have been three forms of T cell antigen receptors (TCRs) identified, α/β (Meuer *et al.*, 1983; Hankins *et al.*, 1983; Samelson *et al.*, 1985), γ/δ (Brenner *et al.*, 1986; Bank *et al.*, 1986) and γ/γ (Alarcon *et al.*, 1987). The vast majority of T cells in normal mice and humans (including all functional Tc and Th cells) bear the α/β type of receptor and therefore it is this receptor that will be discussed here. The functional TCR consists of the α/β heterodimer (Ti) in association with the CD3 (T3) molecular complex, to form the TCR-CD3 complex. It is the disulphide-linked heterodimer of the two highly polymorphic glycoproteins, α and β, which is involved in antigen recognition. Each of these chains consists of variable (V) and constant (C) domains stabilised by intrachain disulphide bonds, a transmembrane region and a cytoplasmic region (Samelson *et al.*, 1985; Hale *et al.*, 1987; Hamme and Glover, 1988). TCRα/β are associated with at least four constant elements of the CD3 complex, γ, δ, ϵ and ζ. These four peptides are thought to traverse the cell membrane and possess a cytoplasmic region (Samelson *et al.*, 1985). A fifth protein, CD3-P21 is transiently associated with the complex (Samelson *et al.*, 1986). It is well documented that Tc use the α and β chains of the TCRα/β to recognise antigen in association with class I MHC antigen on the target cell. For example, transfection of α and β chain cDNAs between T cell clones confirms that the α and β heterodimer confers both antigen and self-MHC specificity upon the cell which expresses it (Dembic *et al.*, 1986). It is outside the scope of this work to examine in detail the role of these and other molecules in T-cell recognition and activation. For a comprehensive review of this area the reader is referred to Terhorst *et al.* (1988).

Antigen processing and presentation

Evidence from early studies of target cell recognition by virus-specific class I restricted Tc strongly favoured the view that class I restricted Tc recognised foreign antigens expressed in their native form on cell surfaces (Sugamura *et al.*, 1977; Gething *et al.*, 1978; Braciale *et al.*, 1978; Kurrle *et al.*, 1979). This model was initially called into question by reports that fragmented forms of specific viral polypeptides could stimulate class I MHC-restricted Tc responses (Guartin and Fan, 1980; Wabuke-Bunoti *et al.*, 1981; Wabuke-Bunoti *et al.*, 1984). Furthermore, other groups report that class I MHC-restricted T lymphocytes recognise expressed products of truncated viral genes (Gooding and O'Connell, 1983; Townsend *et al.*, 1985). For example, Townsend *et al.* (1985) showed that Tc clones specific for the nucleoprotein (NP) of influenza virus were able to recognise and lyse L cells cotransfected with the NP gene and the appropriate class I gene; NP is not expressed on the cell surface. Additionally, Townsend *et al.* (1986) showed that target cells sensitized with synthetic oligopeptides corresponding to specific portions of a viral polypeptide were also recognised by specific Tc. These observations and work by others (Maryanski *et al.*, 1988) led to the hypothesis that class I MHC-restricted Tc, like class II MHC-restricted helper T cells, recognise processed and possibly degraded forms of protein antigens in association with MHC antigens.

There is much interest now on the pathways that lead to class I and class II MHC-restricted presentation. For example, Braciale and coworkers (1987) have examined the presentation of the influenza haemagglutinin (HA) polypeptide to class I and class II MHC-restricted Tc. They found that exogenously introduced HA was not recognised by class I MHC-restricted Tc, but was by class II MHC-restricted Tc (Morrison *et al.*, 1986) and that the presentation of this exogenously introduced HA was sensitive to

chloroquine, an agent which inhibits antigen processing. Work with the protein synthesis inhibitor anisomycin found that class I MHC-restricted Tc require HA *de novo* synthesized in the target cells for sensitization. Thus Braciale *et al* (1987) proposed from this and other work (Morrison *et al.*, 1986), that there are two distinct pathways of antigen presentation to T lymphocytes; the exogenous presentation pathway which is concerned with the presentation of antigens introduced into the presenting cell from without and the endogenous pathway which is concerned with presentation of antigens which are synthesized *de novo* in the presenting cell. Furthermore, a close association between the MHC restriction of an antigen-specific T lymphocyte and the pathway of antigen presentation to that T lymphocyte is suggested, although it is reported not to be absolute. Thus it is proposed, presentation of a given antigen by the endogenous pathway preferentially triggers a response from class I MHC-restricted T lymphocytes directed to that antigen and similarly for the exogenous pathway and class II MHC-restricted T lymphocytes. Braciale and coworkers suggest that interactions between MHC molecules and antigen within the presenting cell may be critical for the demarcation of these routes. For example, it may be that antigen presented by the endogenous route can only associate intracellularly with newly synthesized or recycling class I MHC molecules. However, a greater understanding of the expression, intracellular trafficking and transport of class I and class II MHC molecules in the antigen presenting cell is necessary before the molecular basis for this link can be explained.

When the phenomenon of MHC restriction was first recognised, two models were proposed to account for it. The first involved the recognition of MHC and the antigen as independent events, mediated by two receptors and the second involved a single T-cell receptor which recognises a complex of MHC

with antigen. Recent evidence has now shown that the latter model is correct. It has been reported by several different groups that, for both class I and class II MHC-restricted T cells, a single receptor can be transferred from one T cell to another and confer dual specificity for MHC and antigen (Dembic *et al.*, 1986; Saito *et al.*, 1987). Additionally, it has been shown that the peptide antigens recognised by class II MHC-restricted T cells selectively bind the isolated class II MHC molecules *in vitro* (with affinities in the micromolar range) to form complexes that stimulate T cells specifically (Babbitt *et al.*, 1985; Buus *et al.*, 1986a). Finally, recent crystallographic studies by Bjorkman *et al.* (1987a, 1987b) have revealed a deep groove on the top of the human class I MHC HLA-A2 antigen which is suggested to be the binding site for the foreign peptide that is recognised together with the HLA antigen by the T-cell receptor (Townsend and McMichael, 1987; Parham, 1988). The groove is reported to run between two long α-helices derived from the α1 and α2 domains of the molecule, and the floor of the groove was formed by β-strands also derived from both α1 and α2 domains (see Bjorkman *et al.*, 1987a; Bjorkman, *et al.*, 1987b for structure of MHC class I molecules). The groove is large enough to accomodate peptides of 8 to 20 amino acids in length which is the approximate size of the peptides used by Townsend *et al.* (1986). Significantly, Chen and Parham (1989) have recently demonstrated that the human class I HLA-A2 antigen does in fact directly bind a 14 amino acid fragment of the influenza matrix protein. Similarly, Babbitt *et al.* (1986) showed that class II MHC antigens can also bind peptide fragments. They demonstrated that a 10 amino acid fragment of hen lyzozyme bound directly to the murine class II MHC antigen, H-2IA^{a}. Although the X-ray crystallographic structure of class II MHC antigens has not yet been determined, it is proposed from amino acid sequence analysis that the class II MHC molecules may also have a groove suitable for peptide binding

similar to that in HLA-A2 (Bjorkman *et al.*, 1987a). Many models have been proposed to explain how association between antigenic peptides and MHC molecules occurs. For example, Townsend and McMichael (1987) proposed that the foreign antigen peptides bind class I MHC molecules as they fold and associate with β_2 microglobulin. Examination of these models, however, is outside the scope of this thesis, therefore the reader is directed elsewhere, Germain, 1986; Townsend and McMichael, 1987; Werdelin *et al.*, 1988; Long and Jacobson, 1989.

The initiation of a specific immune response from T-cells is now believed to require not only recognition, by the T-cell receptor, of the antigen in association with the appppropriate MHC molecule, but also antigen-independent interactions between adhesion molecules which enhance the avidity of interactions between T-cells and antigen presenting cells and are possibly involved in signal transduction across the membrane (Springer *et al.*, 1987; van Noesel *et al.*, 1988). Two ligand-receptor pairs of these adhesion molecules have been identified to date. The lymphocyte function associated antigen-1 (LFA-1) and the CD2 receptors, which are expressed on the surface of T-cells, interact with the ligands intercellular adhesion molecule-1 (ICAM-1) or ICAM-2 and LFA-3 respectively, these molecules being expressed on a variety of cell types (Dustin *et al.*, 1986; Springer *et al.*, 1987; Altman *et al.*, 1989; Figdor *et al.*, 1990). It has been shown by Dustin *et al.* (1988) that IFN-γ increases the expression of ICAM-1 on a variety of cell types and it is possible that this increase of ICAM-1 on target cells may augment the avidity of Tc-target cell interaction. In addition, the human cell surface markers CD4 (L3T4 in the mouse) and CD8 (Lyt 2 in the mouse) which, as discussed earlier, have been found to be present on class I and class II MHC-restricted T-cells respectively, are now known to directly interact with the antigen they are restricted to

(Bushkin *et al.*, 1988; Doyle and Strominger, 1987). Thus it is proposed that the interaction of the class I and class II MHC antigens with CD8 and CD4 respectively may also augment the avidity of effector-target cell association.

1.3 The immune surveillance theory

In the 1950s both Thomas (1959) and Burnet (1957) independently proposed what later evolved as Burnet's immune surveillance theory (1970). In its original form, the theory proposed that one function of the immune system was to recognise and destroy nascent neoplastic cells and thus to serve as a mechanism for the control of carcinogenesis. It was predicted by this theory that (1) cancer cells are antigenic, (2) cancer cells can be recognised and destroyed by an immune response and (3) immunosuppression is associated with an increased incidence of cancer. Also implicit in this theory was the assumption that it involved a systemic, antigen-specific, T cell-mediated immune response (Kripke, 1988). There are many experimental systems where all the predictions of the immune surveillance theory are followed. For instance, tumours induced by DNA viruses such as Shope fibroma virus of rabbits (Strayer *et al.*, 1984) and polyomavirus in the mouse (Klein and Klein, 1977), RNA viruses such as murine mammary tumour virus and Moloney sarcoma and leukaemia virus (discussed later in some detail) and those induced by UV radiation (Kripke, 1981). However the theory has received much criticism, the main points of which will be discussed here (for a comprehensive review see Woodruff, 1980).

Hewitt *et al.* (1976) argued that many tumours which arise spontaneously, i.e., without the deliberate administration of known oncogenic agents, in inbred laboratory animals lack demonstrable tumour antigens and thus human tumours which are also spontaneous, will be equally lacking in tumour antigens and therefore will be beyond the reach of immune surveillance. This is now being refuted as many so called 'spontaneous tumours' in humans have been associated with physical and chemical factors and also viral oncogenes (e.g. B-cell lymphomas with Epstein-Barr virus, Kaposi's sarcoma

with cytomegalovirus, hepatocellular carcinoma with hepatitis B virus, various squamous cell carcinomas with human papillomavirus and UV radiation, etc.) all of which induce strongly immunogenic tumours in animals.

It is predicted from the immune surveilllance theory that immunodeficient animals and patients should have higher incidences of tumours, however it is argued by some groups that this is not the case. The main critisism is based on evidence from studies comparing the incidence of spontaneous tumours in immunocompetent and immunodeficient animals. Early studies using athymic nude or thymectomized mice (which show virtually no evidence of any functional T cell activity) showed no significant difference in the overall incidence of spontaneous tumours between nude mice and normal litter mates. However this is of little importance as the life span of these immunodeficient mice was much shorter than that of the normal mice (Custer *et al.*, 1973). Later studies carried out under germ-free conditions have recorded differences in tumour incidence between nude and heterozygous litter mates however the data are inconclusive. Outzen *et al.* (1975) recorded 22 lymphoreticular tumours, which appeared after a mean latent period of 57 weeks, but no other tumours in a population of 261 germ-free nudes (Balb/c background) as against a total of 2 lymphomas among 308 germ-free heterozygous littermates. Stutman (1978), however, argues that this is not significant as the reported incidence of lymphomas in normal Balb/c mice ranges from 1 to over 20 % and furthermore he reports that the incidence of tumours in nude mice from his own studies are comparable to that of the immunologically normal controls. Moreover, in view of the increasing evidence for the involvement of physical, chemical and oncogenic factors in the induction of spontaneous tumours, it now appears that using nude mice to study spontaneous tumour incidences is not relevant to the

human situation as they are not exposed to the necessary environmental factors. Nude or thymectomized mice have also been used to examine the effect of oncogenic viruses, chemical and physical factors. It is reported that nude mice (Stutman, 1975) are more susceptible to oncogenesis by polyoma virus and neonatally thymectomized mice have increased incidence of tumours when inoculated with adenovirus type 12 (Allison *et al.*, 1967). Furthermore, the haplotype of the inbred strain of mice also appears to determine its ability to mount an immune response to a particular virus, as discussed in some detail in section 1.7.

From the human situation there is much evidence that patients suffering from primary immunodeficiency or subjected to prolonged treatment with immunosuppressive drugs show an increase in incidence of tumours (Spector *et al.*, 1978; Penn, 1988). For instance, the incidence of malignant neoplasms in children and young adults with primary immune deficiency disease is 100 or more times greater than the incidence in children of the general population, and all the main forms of primary immune deficiency diseases contribute to this increase (World Health Organization, 1978). The distribution of tumour types in children with primary immunodeficiency differs markedly, however, from that seen in the general population. Woodruff (1980) reports that from the children's immunodeficiency registry 67% of the patients had lymphoreticular tumours and 25% had leukaemia, whereas the corresponding figures derived from death certificates of over 29 000 children in the USA were 8% lymphoreticular tumours, 48% leukaemia and 44% other tumours. Similarly a later study of patients with the immunodeficiency disease Wiskott-Aldrich syndrome reported an increase in the incidence of tumours 100 times that of the general population with 75% of these being non-Hodgkin's lymphoma (Catelingam *et al.*, 1985). More recently with the current epidemic of the human immunodeficiency virus

(HIV) an association is becoming apparent between HIV infection and an increase in the incidence of tumours (Haraly *et al.* 1988; Kaplan *et al.*, 1987; Kaplan *et al.*, 1989; Gail *et al.*, 1991; Rabkin *et al.*, 1991). Rabkin *et al.* (1991) report the incidence of non-Hodgkin's lymphoma and Kaposi's sarcoma has increased over 10-fold and 5000-fold respectively, in single San Francisco men and HIV infection is thought to be the explanation for most or all of these extra cases. Other tumours that may have an increased incidence in HIV-infected patients include Hodgkin's disease, basal cell carcinoma of the skin and squamous cell carcinoma of the anus (Heyer *et al.*, 1989; Biggar, 1990) and most recently breast cancer (Remick *et al.*, 1991).

In the past, the fact that many tumours do progressively grow and kill their hosts was considered evidence against the immune surveillance theory. However in view of all the evidence in favour of immune surveillance it is proposed that many immunogenic tumours are destroyed before their presence is detected and those tumours that survive escape surveillance by the emergence of non-immunogenic clones or by decreasing the general immunological responsiveness of the host. A discussion of the mechanisms that are employed to decrease the general immunological responsiveness of the host are outside the scope of this work. The most obvious way that a tumour cell can become non-immunogenic is by non-expression of its antigens as is observed with Burkitt's lymphoma tumour cells (Rickinson *et al.*, 1989). These tumour cells have been found to be negative for EBNA 2 and LMP, two viral gene products with important effector roles in normal B cell transformation, which appear not to be necessary for the continued growth of the malignant clone. These tumour cells also show low/non-expression of the adhesion molecules which are critical for target cell conjugation with effector T cells thus suggesting another possible mechanism for escaping

surveillance. As discussed in detail in section 1.2 T cells recognise foreign antigens in association with self MHC antigens thus by down-regulating the level of expression of MHC antigens a tumour cell could evade recognition. The evidence supporting the occurrence of this effect is examined in the following section.

1.4 The regulation of class I and class II major histocompatibility antigens by viruses and activated oncogenes

It has recently been shown by several groups that some viruses are able to affect host cell MHC antigen expression. Clearly this is significant because, as discussed before, the T cells of the host animal's immune system require MHC antigens in order to respond to viral antigens. For a comprehensive review of virus modulation of host cell MHC antigen expression the reader is directed to Maudsley *et al.* (1989).

Down-regulation of class I antigen expression

Several viruses have been reported to down regulate class I MHC antigen expression; herpes simplex virus type 1 (HSV-1) and HSV-2 (Jennings *et al.*, 1985), HIV-1 (Schappler *et al.*, 1989), adenovirus 12 (Ad12) (Schrier *et al.*, 1983; Ackrill and Blair, 1988) and Ki MSV and MLV down-regulate induced class I antigen expression (Maudsley and Morris, 1989a) and it is postulated that this may be beneficial to the virus as a mechanism for escaping immune recognition. In fact, for Ad12 the down regulation of host cell class I expression by the E1A gene product has been found to correlate with the tumourigenicity of the virus-infected cells (Bernards *et al.*, 1983). Furthermore, introduction of a functional class I gene into a highly tumourigenic Ad12-transformed cell line resulted in complete loss of oncogenicity at certain tumour cell doses (Tanaka *et al.*, 1985). Similarly restoring class I antigen expression on Ad-12 transformed cells with IFN-γ also abolishes tumourigenicity (Hayashi *et al.*, 1985).

The mechanisms whereby viruses reduce MHC expression are generally not clear, however it has been found by Lyons *et al.* (1987) that the adenovirus E1A gene products go to the nucleus where they affect transcription

(Schrier *et al.*, 1983) or transcriptional processing (Vaessen *et al.*, 1987) of class I genes. Also, Andersson *et al.* (1985) have shown that the adenovirus E3 gene product E3/19K binds the MHC class I α chains, preventing their going beyond the Golgi and hence from being expressed.

Maudsley and Morris (1989a) suggest that as down-regulation occurs for both Ki MLV and MSV, the most likely explanation is that some sequence in the LTR which is present in both MSV and MLV acts in *trans* to regulate class I expression. *Trans* active IFN response sequences (IRS) which suppress responses to IFN in hepatitis B virus (HBV) (Thomas et al., 1986; Onji et al., 1989) are also found to be present in the LTRs of Ki MLV and MSV. Friedman and Stark (1985) noted a potential IRS on the basis of conserved sequences in the 5'-flanking regions of several genes responsive to α/β IFNs, for instance, human class I HLA, metallothionine (for consensus sequence see Figure 1.4) and similar sequences have also been found in association with other IFN-inducible genes e.g. H-2 in the mouse (Kimura *et al.*, 1986). In the case of H-2 the IRS has been shown to be functionally involved in the induction of α/β IFNs. Further, Reid *et al.* (1989) report the existence of a IFN-stimulable response element (ISRE) in the 5' flanking region of the 9-27 gene which appears to be responsible for the inducibility of this gene with IFN-γ; homologous ISRE also present in 6.16, ISG 15 and ISG 54 genes which are predominantly inducible by IFN-α,β (consensus sequence shown in Figure 1.4). It can be seen that the proposed core sequences of IRS and ISRE are the same although the sequence outside the cores are different. Further it can be seen that the core sequence of IRS and ISRE is found in the LTR of Ki MLV and MSV as mentioned above and, interestingly, in the LTR of Mo MLV and MSV, however it is unknown as yet whether these IRS have any functional activity.

Figure 1.4. IRS and ISRE consensus sequences

	C T
Consensus IRS	TTCWG WACCT CWGCA GTTTC TCCTC T-CT
core	A GTTTC
Consensus ISRE	AGTTTCWWTTTCC
core	AGTTTC
Ki-MSV LTR	GCUAA AACAA CAACA GUUUC AAGAG ACCC
Ki-MLV LTR	GCUAA AACAA CAACA GUUUC AAGAG ACCC
Mo-MSV LTR	GUCCA GCCCU CAGCA GUUUC UAGUG AAUC
Mo-MLV LTR	GUCCA GCCCU CAGCA GUUUC UAGAG AACC

(Adapted from Maudsley and Morris, 1989b; Norton *et al.* 1984a).

Clinical observations of certain malignant tumours, including teratocarcinomas (Jacob, 1977), eccrine porocarcinomas (Holden *et al.*, 1984), cervical carcinomas (Sanderson and Beverley, 1983) and melanomas (Versteeg *et al.*, 1989) have also shown greatly reduced or undetectable levels of class I antigens on the cell surface (in contrast to their normal cellular counterparts) thus it is argued that this is a possible mechanism for the escape of certain tumours from T cell-mediated immune surveillance and suggests future therapeutic approaches for the reversal of certain malignancies (Tanaka *et al.*, 1985).

Up-regulation of class I antigen expression

Nevertheless, down-regulation of MHC class I genes is not a universal characteristic of malignant tumours; Dahlof (1990) reports that in the polyomavirus-induced SEWA tumour the enhancement of the tumourigenicity caused by c-*myc* amplification is not mediated through down-regulation of MHC class I antigens. Up-regulation has also been observed; Carlow *et al* (1989) reports the isolation of murine mammary adenocarcinoma variants which, after *in vitro* treatment with the DNA-hypermethylating agent 5-azacytidine (5-aza), express elevated class I MHC antigens relative to the level of expression in the parent tumour and are rejected in syngeneic mice by a T-cell-dependant process. To ascertain whether elevated immunogenicity is a function of increased class I expression, high class I expressers were isolated using the fluorescence-activated cell sorter (FACS). It was found that these variants displayed unaltered tumourigenicity in immunocompetent syngeneic mice, therefore Carlow *et al.* (1989) conclude that phenotypic changes other than, or in addition to, elevated class I antigen expression cause the reduced tumorigenicity of immunogenic tumour variants.

Lymphomas in AKR mice (H-2^k haplotype) also show a significant increase in total class I antigens on the cell surface (Kawashima *et al.*, 1976), as do radiation leukaemia virus (RadLV) induced thymomas (Maruelo *et al.*, 1978). Schmidt et al. (1981) showed that the level of D^k expression in AKR lymphomas was greatly increased whereas K^k expression usually remained unchanged. However, in the case of an AKR leukaemia cell line with reduced levels of K^k but increased levels of D^k tumourigenicity was abolished with the transfection of a functional K^k gene. Increased levels of class I MHC antigen expression were also observed by Flyer *et al.* (1985) after infection of BALB/c-3T3 fibroblasts with Mo MLV. It was reported that fibroblasts infected with Mo MLV exhibit up to a tenfold increase in cell surface expression of all three class I antigens (K, D and L). And yet, when BALB/c-3T3 fibroblasts were coinfected with Mo MLV and Mo MSV, no increase in the level of class I antigen expression was observed, thus it is suggested that Mo-MSV exerts an inhibitory effect on the Mo MLV induced enhancement of class I antigens. It is also postulated that this inhibition is specific for Mo MLV enhanced class I expression as MSV infection does not inhibit the enhancement of class I antigens by IFN-γ (Flyer *et al.*, 1985). In contrast, are the previously discussed results of Maudsley and Morris (1989a) which show that the closely related Ki MLV, in fact, diminishes the induction of class I antigens by IFN-γ to a similar extent as Ki MSV. This up-regulation of class I MHC antigen expression by M MLV which has been shown to possess an IRS-α may be significant when considering the role of IRS-α in class I expression.

Down-regulation of class II antigen expression

Several viruses have also been shown to down-regulate class II MHC antigen expression, Rous sarcoma virus (RSV) (Powell *et al.*, 1987) and Ki MSV (Maudsley and Morris, 1988). Maudsley and Morris report that Ki MSV but not

Ki MLV in fact largely abolishes IFN-γ induced class II H-2A (Maudsley and Morris, 1988) and H-2E (Maudsley and Morris, 1989b) antigen expression in the murine fibroblast line C3H10T½. They suggest that the abolition is due to the genomic region of Ki MSV not shared with Ki MLV, that is, the v-Ki-*ras* gene. The more recent study suggests this abolition is not due to transformation-induced changes in the kinetics of the response to, or in the sensitivity of the cells to, IFN-γ.

Up-regulation of class II antigen expression

Measles virus, JHM coronavirus and simian immunodeficiency virus have all been shown to induce class II MHC antigen expression on their host cell (Hanna *et al.*, 1986; Hanna *et al.*, 1987b; Kannagi *et al.*, 1987) and it is suggested this may lead to inappropriate responses, for example, to autoantigens in tissues where levels of MHC antigens especially class II are normally low or absent. This is found to be the case for allergic encephalomyelitis in rats, where an immune response is raised against the myelin basic protein (Hahn *et al.*, 1987a; Hanna *et al.*, 1987b).

1.5 What are the effector mechanisms in responses to tumours

Since it was shown that cytotoxic T cells (Tc) can specifically lyse target cells expressing tumour and histocompatibility antigens or foreign MHC antigens it has often been assumed that they would be the effector cells in rejection responses (Wagner *et al*, 1980). There is much evidence to support the involvement of Tc, however it has more recently become apparent that other T cell subsets and/or mechanisms may also be involved (for review see Robins, 1986). Even though it has been shown by several groups that tumour specific Tc activity can be found in spleen or lymph node cells from mice immunized with cells or those in which a tumour has regressed, it is argued by some workers that Tc responses are not absolutely required for tumour rejection (Fujiwara *et al*., 1984; Greenberg *et al*., 1985). In fact, in some tumour models, no Tc activity has been detected in spleen cells from immunized mice. This was found to be the case for a chemically induced murine hepatoma and a cloned cell line of the methylcholanthrene induced fibrosarcoma (Fujiwara *et al*., 1987). It was shown that these tumour cells are highly susceptible to attack by activated macrophages or lymphokine-activated killer cells and also a Lyt2$^-$ T-cell subset isolated from spleen cells of tumour-immune mice could eradicate the tumour. It is suggested by Hamaoka and Fujiwara (1987) that when tumours are highly immunogenic and tumour-specific immunity can be easily induced, Tc may be less important in rejection responses than has been thought. Studies to investigate the anti-tumour mechanisms of Lyt-1$^+$2$^-$ T cells in the absence of detectable Tc activity have been carried out by Fujiwara *et al*. (1985). They concluded that the tumour-specific Lyt-1$^+$2$^-$ T cell subset which is known to produce the macrophage-activating factor, now known to be IFN-γ, exert their effect by producing a lymphokine(s) which activates the nonspecific tumouricidal effector macrophages.

The Lyt-2 antigen was originally identified as a Tc-specific marker, however as mentioned in section 1.2, other workers (Swain *et al.*, 1979; Mizuochi *et al.*, 1986) have shown that some Lyt-2$^+$ T cells may function as helper cells similar to L3T4$^+$ cells. Mizuochi *et al.* (1986) showed that a subset of Lyt-2$^+$ T cells functioned as helper cells in the activation of histocompatible Tc precursors in conventional class I allospecific Tc responses. They also report that this helper activity is confirmed by their ability to produce interleukin 2 (IL-2). Hamaoka and Fujiwara (1987) report a Lyt-2$^+$ T cell subset which also seems capable of exerting its anti-tumour effect by producing lymphokines such as IFN-γ and activating non-T cell effector mechanisms. Hamaoka and Fujiwara (1987) discuss the possible significance of these mechanisms *in vivo* when a tumour mass may consist of cells expressing quantitatively and/or qualitatively different tumour antigens. It is argued that if tumour-specific T cells such as Tc are generated and attack directly as the sole anti-tumour effector mechanism, less immunogenic or non-immunogenic tumour cells coexisting in each tumour mass will escape from the specific immune attack of the host (as discussed earlier as a likely mechanism of escape from immune surveillance). They postulated that a pathway initiated by tumour-specific T cells but in which non-specific tumouricidal effector cells are activated could eradicate less immunogenic tumour cells as well, because tumour cell killing would not require the specific recognition of tumour antigens at the effector phase.

There is much evidence for the cooperation of different cell types and mechanisms in tumour regression. However, the majority of studies still favour T cells, particularly Tc as the main cell type responsible for the elimination of tumour cells, especially in cases of virally or chemically induced tumours which are highly antigenic (Doherty *et al.* 1981). The most convincing evidence supporting the involvement of Tc as the primary immune

response to tumour cells comes from adoptive transfer experiments (Mulé et al., 1987; Shu *et al.*, 1987a). Shu *et al.* (1987b) showed by *in vivo* T cell subset depletion with monoclonal antibodies that the primary effector cells in regression of the MCA 105 tumour are Tc expressing the Lyt-2 phenotype. They also showed that these effector cells could be expanded in numbers *in vitro* with continuous stimulation by tumour cells in the presence of IL-2. This has also been achieved by Plata and colleagues (1975) in the Mo MLV/MSV system. They showed that Tc specific for syngeneic MLV/MSV tumour cells are generated in mixed lymphocyte tumour cell cultures. Additionally, they found that spleen cells from mice having rejected a Mo MLV/MSV tumour were able to generate Tc response 10-fold higher than that of normal spleen cells. Similarly, Mulé *et al* (1987) showed that Lyt-2$^+$ cells are predominant effectors in the elimination of both pulmonary micro- and macrometastases and that the depletion of L3T4$^+$ cells had no effect on tumour regression.

More evidence of the importance of Tc in tumour immune recognition comes from studies which show a correlation between the down-regulation of MHC class I antigen expression and increased tumourigenicity. As was addressed in the section 1.2, the majority of Tc are class I restricted thus it is concluded that the increased tumourigenicity in many cases may be a result of the tumour cells escaping recognition by the cytotoxic effector cells due to a decrease in the expression of host class I proteins. For more detail account of down-regulation of class I and class II MHC antigens by viruses and other factors e.g. oncogene activation as a mechanisms for evading immunological recognition see the previous section.

The information already available on tumour regression in the murine sarcoma virus/murine leukaemia virus (MSV/MLV) system suggests that Tc play

a major role (Plata and Sordat, 1977; Gillespie *et al.*, 1977; Leclerc and Cantor, 1980). Although the other mechanisms discussed earlier in this section may also be involved, this study will be concerned mainly with the importance of the Tc component of the immune response in MLV/MSV tumour regression. In section 1.7 the data presented to date concerning the immune responses and the target proteins involved in the regression of MLV/MSV are reviewed.

1.6 A review of murine leukaemia and sarcoma virus biology

An introduction to retroviruses

Retroviruses are RNA viruses which replicate through a DNA intermediate. This retroviral replication will be described in the following passages, however, first a brief introduction to the MLV proteins will be given. For a more detailed account of both, the reader is referred to Weiss *et al.* (1985) and Coffin (1990).

The three viral genes necessary for replication are *gag* (group specific antigens), which encodes the capsid proteins, *pol* which encodes the reverse transcriptase (polymerase) and *env* which encodes the viral envelope proteins. There are four internal structural proteins encoded by *gag*; the major capsid polyprotein of 30 000 daltons (p30), a hydrophobic protein of 15 000 daltons (p15), a phosphoprotein of 12 000 daltons (pp12) and a basic protein of 10 000 daltons (p10). The physical order of the proteins within the *gag* gene product is NH_2 - p15 - pp12 - p30 - p10 - COOH and they are derived by proteolytic cleavage of the nonglycosylated precursor polyprotein $Pr65^{gag}$. There are also glycosylated forms of the gag precursor proteins ($gPr85^{gag}$ and $gPr95^{gag}$) which are expressed on the surface of virus infected cells but not found in virus particles (Weiss *et al.*, 1984).

There are two viral envelope proteins encoded by *env*; a glycoprotein of approximately 70 000 daltons (gp70) and a nonglycosylated protein of 15 000 daltons (p15E). The order of gp70 and p15E within the env gene product is NH_2 - gp70 - p15E - COOH. These proteins are synthesized as a glycosylated protein precursor, $Pr85^{env}$ or $Pr95^{env}$, the cleavage of which is carried out before virus assembly. gp70 is the major surface component of the virus and

is involved in the adsorption of viral particles to cells. Both gp70 and p15E are exposed on the external surface of the viral particle; gp70 comprises the virus knobs whereas p15E is more closely associated with the membrane. gp70 molecules interact with cell-surface receptors which initiates uptake of the viral particle into the cell (as discussed later). p15E is reported to be immunosuppressive in that it inhibits the immune responses of lymphocytes, monocytes and macrophages (Cianciolo *et al.*, 1985). It has been proposed by Copelan et al. that p15E exerts its immunosuppressive effects by blocking the production of interleukin 2 by lymphocytes (Copelan *et al.*, 1983). The last gene required for replication is *pol* which encodes the 70 - 80 K dalton reverse transcriptase (DNA polymerase), the integrase protein needed for integration of the viral into cell DNA and a protease responsible for the cleavage of the *gag* and *pol* polyproteins. The reverse transcriptase molecule, as discussed below, has two main enzyme activities; a DNA polmerase capable of copying RNA or DNA templates and a ribonuclease active on RNA:DNA hybrids (RNAse H).

The retroviral replication cycle, which is schematically outlined in Figure 1.6a, can be divided into five stages. The first stage includes the adsorption, penetration and uncoating of the virions. The cell-surface receptor molecule for Mo MLV, which interacts specifically with the gp70 protein on the virion envelope, has recently been identified, however the site of interaction is unknown. The mechanism by which retroviruses enter cells is one of the most poorly understood aspects of the virus life cycle. It seems that they are internalized via receptor-mediated endocytosis followed by fusion of viral envelope and endosomal membrane, however again little is known about the fusion itself. The fate of the various capsid proteins upon fusion is not clear although it is assumed that the reverse

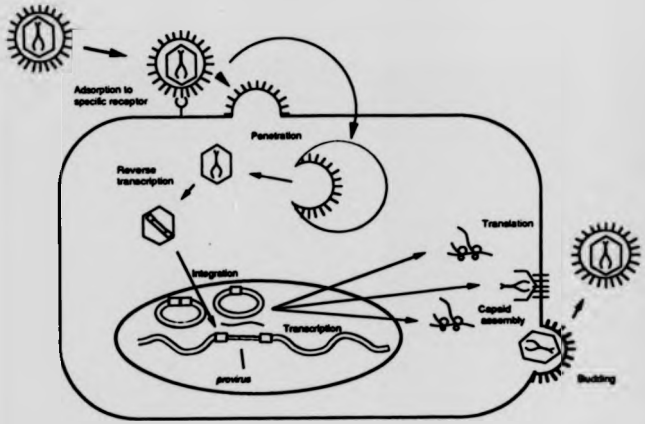


Figure 1.6a. An overview of retrovirus replication (Fields *et al.*, 1990).

transcriptase, integrase, p10, and p30 remain with the genome, whereas p15 may remain associated with the membrane (Coffin, 1990).

Stage two involves the synthesis of the unintegrated (free) viral DNA from the genomic RNA using the enzymes that entered the cell in the virion. This process is summarized in figure 1.6b. The initial DNA strand synthesized complementary to the viral RNA is called the negative strand. The negative strand is synthesized as a single continuous piece of DNA. Its synthesis occurs, however, in three discrete steps because this strand is synthesized from three different templates. DNA synthesis initiates by elongation from the 3' end of the primer tRNA through U5 and the R sequence to the 5' end of the genome. When elongation of the negative strand reaches the 5' end of the RNA template, it stops because it has run out of template. This delay in further DNA synthesis is seen *in vivo* and *in vitro*. The resulting DNA molecule which is a copy of the short region consisting of R and U5 between the the primer-binding site (PB) and the 5' end of the genome is called the *strong stop* DNA. Once strong stop DNA is made, the newly made strand must transfer, presumably with the reverse transcriptase, to the other end of the genome in order for synthesis to continue. This jump is facilitated in several ways; first, the R sequences which are duplicated at each end of the genome allowing appropriate base pairing to direct continued synthesis. Second, the RNase H activity of reverse transcriptase removes the newly copied RNA, leaving the DNA free to pair with the R sequence at the other end. Finally, it appears that some unidentified aspect of the capsid structure is necessary for this process, as the jump has never been accomplished *in vitro* with purified enzyme and template. Once the jump has occured, synthesis of the negative strand can proceed unchecked to the 5' end of the template, which is now the 5' end of PB because R and U5 will have been removed earlier by RNase H. The molecule that results from

copying the genome is a complete, but slightly permuted, complement of the genome with the tRNA primer still attached at the 5' end (Coffin, 1990).

To obtain a completed molecule, it is necessary for reverse transcriptase to copy the negative-strand DNA molecule just made. This process also requires a primer molecule to initiate synthesis. The 3' end that serves this role does not preexist, it must be created. This is accomplished by a specific cleavage of the RNA template at the 5' end of U3. This specific cleavage appears to occur by a variation of the RNase H reaction (Coffin, 1990). Following initiation, elongation of the positive strand is carried out by reverse transcriptase toward the 5'end of the negative strand. When the end is reached, as with the negative strand, a jump must occur to permit complete synthesis. The redundant sequences used in this case are formed by the copy of the primer-binding site at the 5' end of the negative strand. The positive strand is elongated by copying the negative strand DNA through the U3, R, and U5 regions and into the primer itself (see figure 1.6b). By copying the first 18 bases of the primer, a direct copy of the primer binding site is yielded which can form yet another template-primer pair with its complement at the 3' end of the negative strand. Completion of the full-length, double-stranded DNA can now be accomplished by the synthesis of each strand to its end (see Figure 1.6b) and the removal of the primer tRNA presumably by RNase H (Coffin, 1990). It can be seen that the ends of this DNA molecule have an extra copy of sequences present only once in the RNA genome. These extra sequences together form a structure called the long terminal repeat (LTR) which, as discussed elsewhere, contains virtually all the *cis*-acting sequences necessary for integration of this DNA to form the provirus, and the expression of this provirus. It is the proviral DNA produced at this stage that is utilised in the construction of retroviral vectors.

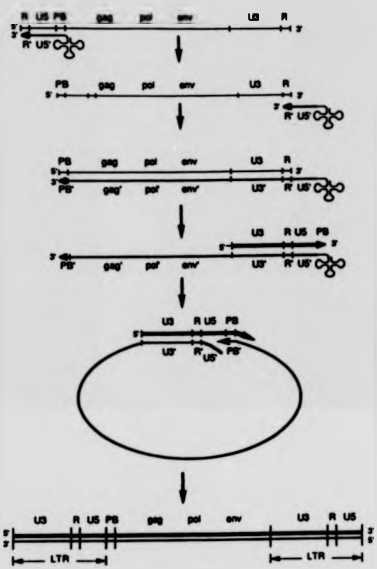


Figure 1.6b. Mechanism of viral DNA synthesis. Thin lines depict RNA, medium lines depict negative-strand DNA, and thick lines denote positive-strand DNA. Negative-sense sequences are also indicated by a "prime" (Fields *et al.*, 1990).

The third stage of replication involves the transit of this DNA, still associated with the incoming virion proteins, to the nucleus and the integration of this viral DNA to form the provirus. The process of integration is still under debate, however the following pathway has been proposed from the current evidence reviewed in Coffins (1990). Following viral DNA synthesis, the core structure containing the linear DNA and the p30 and integrase proteins (and possibly the reverse transcriptase and p10) enter the nucleus. The 3' terminal two bases (usually AA) at either end are removed by the integrase enzyme, leaving a 3' OH end. A staggered cut is introduced into the target DNA with 4-6 bases of overhanging 5' phosphorylated end and this end is joined to the 3' ends of the viral DNA. A cellular DNA repair system fills in the resulting gap in the molecule, displacing the two mismatched bases at the 5' end of the provirus and ligating the remaining ends. This gap repair of the initial staggered cut generates the characteristic duplication of cell DNA flanking the provirus (Coffin, 1990).

Stage four involves the synthesis of viral RNA by cellular RNA polymerase II using the integrated provirus as a template, the processing of the transcripts to genome and mRNAs and the synthesis of the virion proteins. A major function of the LTR is to provide signals recognised by cellular transcription machinery for the efficient expression of the provirus. Transcription of the provirus is initiated at the site where the capping group is placed (U3-R junction) and it apparently proceeds through the 3' LTR into the flanking cell DNA, with the final 3' end determined at the end of R by cleavage and polyadenylation. All retrovirus genomes are synthesised by the same enzyme responsible for synthesis of cell mRNA; moreover, the LTRs of different retroviruses all contain sequences identifiable with corresponding sequences in normal cellular genes.

although often in complex combinations (Coffin, 1990). Virtually all of these sequences lie upstream of the initiation site of transcription and thus they lie within U3. Sequences essential for transcription, such as TATA and CCAAT boxes are found in the U3 region. Furthermore, in the enhancer region found in the 5' 70 base pair repeat of the LTR, seven binding sites for six different transcription factors have been identified (Speck and Baltimore, 1987).

The provirus is transcribed into a single RNA precursor, which is subsequently processed by a) polyadenylation at the 3' end to yield a genome-length molecule and b) splicing of a fraction of the transcripts to generate at least one subgenomic mRNA species. After transport to the cytoplasm, a fraction of the full-length RNA is reserved for genomes and the remainder is used as mRNA for *gag* and *pol*. The *gag* gene translated from the full-length RNA yields a precursor polyprotein that is subsequently cleaved to yield four capsid proteins as described at the beginning of this section. The *pol* gene product is synthesized as a large *gag-pol* fusion protein ($pr180^{gag-pol}$) by read through of a amber stop codon. The spliced RNA is translated on membrane-bound polyribosomes to give the *env* precursor protein (see MLV proteins above).

Finally, the fifth stage involves the assembly and budding of virion, and the processing of the capsid proteins. This stage of replication is still poorly understood. Current opinion is based on a model first proposed over 10 years ago by Bolognesi *et al.* (1978), however discussion of this model is outside the scope of this report.

A brief introduction to the Mo and Ki murine leukaemia and sarcoma viruses

The Mo and Ki MLV are weakly oncogenic Type C retroviruses which, as will be described later, have been shown to be highly immunogenic . The Ki virus causes erythroblastosis in mice (Kirsten, W. *et al.*, 1967) and also induces lymphomas in rats and mice which are indistinguishable from those induced by Gross murine leukaemia virus (Kirsten, W. and Mayer, L., 1967). The Mo virus, upon inoculation into new-born mice, gives rise to leukaemia of mostly T cell origin (Gross, 1970).

The highly oncogenic Ki MSV was derived from Ki MLV during growth of the leukaemia virus in a rat (Kirsten, W. and Mayer, L., 1967). Analysis of the Ki MSV genome has shown that it arose from recombination between the leukaemia virus and two rat-derived components; the Ki-*ras* oncogene which confers the increased oncogenicity of the virus and virus-like 30S (VL 30S) which is a member of a class of retrovirus-like genetic elements(Shih *et al.*, 1978; Ellis, R. *et al.*, 1981). The *ras* gene product is discussed in some detail in the following section.

Mo MSV was isolated from a tumour that developed in a BALB/c mouse after injection with Mo MLV (Maloney, 1966). The Mo MSV genome appears to have arisen by a complex recombination event between the injected Mo MLV and mouse cellular genetic material. It has been shown that Mo MSV retains about 2.2kb of MLV sequences at its 5' end and about 0.7kb of MLV sequences at its 3' end (Reddy *et al.*, 1980; Van Beveren *et al.*, 1981). In addition, the 3' end of the Mo MSV genome contains 1157 contiguous nucleotides that are not present in Mo MLV but can be identified in the DNA of uninfected mouse cells (Frankel and Fischinger, 1976; Tronick *et al.*, 1979; Jones *et al.*, 1980). These viral sequences which were acquired during the

recombination event that gave rise to Ha MSV are defined as v-Ha-*mos*. Andersson *et al* (1979) found that a restriction fragment of Ha MSV DNA synthesized *in vitro*, which includes the *mos* sequence, efficiently transforms NIH 3T3 cells. The protein product of the *mos* gene of Ha MSV appears to be a 37 KD polypeptide called $p37^{mos}$.

The *ras* gene product

The *ras* oncogene (Ha, Ki and N) has been shown to encode a phosphoprotein of 21 kD ($p21^{ras}$) (Shih *et al.*, 1979), which is known to be localized on the inner surfaces of the plasma membrane of transformed cells (Willingham *et al.*, 1980). It has been found that normal $p21^{ras}$ is a GTP binding protein (Scolnick *et al.*, 1979; McGarth *et al.*, 1984; Comerford *et al.*, 1989) which undergoes autophosphorylation of the threonine residue at position 59. It is postulated that this GTP binding activity and the association of $p21^{ras}$ with the inner membrane strongly suggests that *ras* gene products are components of signal transduction mechanisms involved in cellular growth control. The current model is that the *ras* proteins become activated upon stimulation, transduce the signal to some effector molecule and subsequently become inactivated. It is argued that mutated *ras* proteins have lost the ability to become inactivated and thus stimulate growth or differentiation autonomously (Ross, 1989). A protein has been found that is involved in the hydrolysis of the GTP bound to *ras*. This protein, GTPase activating protein (GAP), binds to the effector domain of the *ras* proteins and might play a role in the transduction of signals from *ras* further downstream of the protein (Calés, 1988; Vogel, 1988; McCormick, 1989). Alternatively, effector proteins not yet discovered might compete with GAP for the effector site (McCormick, 1989). In addition, studies by Gibbs *et al.* (1984) on *E. coli* expressed proteins showed that transforming *ras*

proteins possess slower GTP-hydrolysing activity than their normal counterparts. Many groups have shown that the *in vitro* transforming properties of v-*ras* oncogenes on established cell lines are due to point mutations at amino acid positions 12, 13, 59, 61 and 63 (Capon *et al.*, 1983; Shimizu *et al.*, 1983; Diamond *et al.*, 1988), which are also found in a variety of tumours. It has been found that these mutations in *ras* proto-oncogenes occur in approximately 90% of pancreas adenocarcinoma, 60% of thyroid follicular carcinoma, 50% of colon adenocarcinoma, 40% of myeloid leukaemia, 40% of seminoma, 30% of lung adenocarcinoma, 30% of liver carcinoma, 30% of multiple myeloma and 20% of melanoma (Bos, 1989; Cheever *et al.*, 1990). These point mutations have been shown to partly correlate with the impaired GTPase activity (Gibbs *et al.*, 1984; Lacal *et al.*, 1986). Work to investigate the importance of these mutations on oncogenicity carried out by this research group will be discussed later.

1.7 Immune responses to MSV/MLV antigens

It has been shown that inoculation of mice with cells expressing Mo MSV/MLV antigens results in the induction of tumours which spontaneously regress. This tumour regression was thought to be a result of an immune response to the MLV antigens, as tumours of cells expressing MSV alone show no such regression (Levy and Leclerc, 1977). Bateman and Morris (personal communication) have found this same tumour regression pattern in the Ki MSV/MLV system. More recently, data supporting the involvement of MSV antigen(s) in tumour regression has been reported. Evidence supporting an immune response to MLV or MSV antigens will be examined in this section.

The importance of murine leukaemia virus proteins in tumour regression

Early studies into tumour regression in the Mo MSV/MLV system demonstrated Tc specific for the Mo MSV/MLV induced tumours in the spleen, lymph nodes, blood and tumours of mice that rejected the tumour (Plata *et al.*, 1976; Holden *et al.*, 1976; Plata and Sordat, 1977; Gillespie *et al.*, 1977). Further analysis of these Tc showed them to be specific for Mo MLV not MSV antigens (Gomard *et al.*, 1978). These Mo MLV specific Tc have been generated *in vitro* by stimulating spleen cells from normal mice or from mice that have rejected their sarcomas, with irradiated Mo MLV-induced lymphomas in mixed lymphocyte tumour cell culture (MLTC) (Carottini *et al.*, 1974; Plata *et al.*, 1975; Weiss *et al.*, 1980). Similarly, adoptive transfer of Tc from mice which have rejected a MSV/MLV induced tumour has been shown to protect irradiated recipient mice from a challenge of MLV infected lymphoma cells but not from MSV infection (Leclerc and Cantor, 1980). It was concluded from this evidence that the immune response, mounted by these Tc *in vivo*, is responsible for the tumour regression in this Mo MSV/MLV

system. As a consequence many experiments were designed to examine this response, the results from which are reviewed in the following sections. Although less work has been carrried out on the Ki MSV/MLV system the same tumour regression pattern has been observed, thus suggesting that the immune response to MLV proteins in this system is also important in tumour regression.

Tc recognition of Moloney murine leukaemia virus proteins

Tc recognition of the envelope proteins

Several groups have suggested that the envelope glycoprotein is recognised by Tc (Kinjuanne *et al.*, 1979; Collins *et al.*, 1980). Recent studies to evaluate the role of the Mo MLV envelope proteins in Tc recognition have used BALB/c derived cell lines (H-2^d) that express the *env* gene products in the absence of the other MLV proteins in a similiar way to this study. The cell lines produced by Flyer *et al.* (1983) express all the *env* gene products (pr80^{env}, gp70 and p15E) and the bacterial neomycin resistance gene (phosphotransferase APH-(3')-I) which confers resistance to the antibiotic G418, geneticin sulfate (Southern and Berg, 1982). Clones from these cell lines were shown to be recognised by syngeneic Mo MLV specific Tc (H-2^d) in *in vitro* lymphocyte-mediated cytotoxicity assays (Flyer *et al.*, 1983; Flyer *et al.*, 1985). Additionally, it was found that the level of expression of the envelope glycoprotein, as determined by fluorescence activated cell sorter (FACS) analysis, showed a direct correlation with the degree of Tc lysis (Flyer *et al.*, 1985). It should be noted that the cell lines produced by Flyer and colleagues also express the product of the neomycin resistance gene which, as discussed in more detail in the final section, may effect the response to the Mo MLV antigens.

Tc recognition of the *gag* encoded proteins

Several groups have suggested the existence of Tc populations specific for *gag* gene products (Gorczynski and Knight, 1975; Green, 1980; Plata *et al.*, 1983). Gorczynski and Knight (1975) reported that in the Mo MSV/MLV system, Tc sensitized *in vitro* to purified antigens with group specificity (*gag* encoded products) but not viral envelope antigens are capable of protecting the irradiated MSV/MLV inoculated animals. Green (1980) working with the AKR/Gross leukaemia virus demonstrated the existence of H-2 restricted Tc specific for the Gross cell surface antigen (GCSA). In addition, Plata *et al.* (1983) have reported that monoclonal antibodies that recognise the p30 determinant can inhibit the cytolysis of Gross MLV infected cells using a mixed population of Gross MLV-specific Tc. However, although this inhibition of Tc activity was investigated against Friend, Rauscher, Moloney, and Gross MLV-infected cells, inhibition of cytolysis was found only against Gross MLV-infected cells, even though the hybridomas producing the anti-p30 monoclonal were derived from mice immunized with Friend MLV.

More recent studies into the role of Tc recognition in the Mo MLV system have produced contradictory results. Plyer *et al.* (1985) transfected the BALB/c-3T3 (H-2^d) murine fibroblasts with the neomycin resistance gene and a cloned Mo MLV *gag* gene to produce a cell line that expresses the precursor protein $pr85^{gag}$ and its glycosylated product $gP85^{gag}$. The precursor protein is not cleaved into the four virion core proteins p15, p30, p12 and p10, as in the case of MLV infected cells. This lack of processing of the precursor is due to the absence of the viral protease encoded in the 5' portion of the *pol* gene which was not included in the expression vector (Crawford and Goff, 1985). These transfected cells were not lysed by Mo MLV specific Tc generated in BALB/c (H-2^d) when used as targets in *in vitro* lymphocytes-mediated cytotoxicity assays, although the

cell surface expression of *gag* on these cells was equal or greater than that detected on the surface of Mo MLV infected cells, as determined by FACS analysis. Flyer *et al.* (1985) suggest from this study that *gag* determinants do not function as Tc target recognition structures. However, recent characterization of three Tc clones specific for $gP85^{gag}$ seem to contradict this study (van der Hoorn *et al.*, 1985). Van der Hoorn and colleagues (1985) characterized these clones, which were generated in C57BL/6 ($H\text{-}2^b$) mice, by the use of specific antisera against the *gag* and *env* polypeptides of Mo MLV and Abelson-X MLV. Cells infected with the latter are deficient in the synthesis of $gP85^{gag}$ but expressing all other Mo MLV encoded proteins. As Abelson-X MLV infected cells were not lysed by these virus-specific Tc clones it is believed that the $gP85^{gag}$ is the protein recognised as opposed to the *env* encoded proteins, gp70 and p15(E), as they are expressed to the same level in Abelson-X MLV and Mo MLV infected cells. It is reported that these clones can protect mice against virus-induced tumours (van der Hoorn *et al.*, 1985) and can induce the destruction of Mo MLV induced lymphoma cells after intravenous injection (Rogers *et al.*, 1984).

Flyer and colleagues (1987) proposed that this discrepancy of results between the two groups may be due to the fact that van der Hoorn *et al.* (1985) worked with Tc clones as opposed to the studies by Flyer *et al.* (1985) which used bulk Tc populations. Perhaps the frequency of *gag*-reactive Tc is much lower than Tc specific for *env* and are thus not detected in a bulk culture cytotoxicity assay. Alternatively, maybe Flyer *et al.* (1985) failed to generate *gag*-specific Tc in their secondary *in vitro* stimulation protocol. More recent evidence on MHC restricted Tc recognition of MLV antigens, which is examined in some detail in the next section, suggest other considerations which may be significant. An

important difference between these two investigations is the haplotype of the mice used, H-2^d and H-2^b for Flyer *et al.* (1985) and van der Hoorn *et al.* (1985), respectively. It now appears possible that the discrepancy of these results may due to the fact that the *gag* gene product produced in the study by Flyer and colleagues is not recognised as it is presented in association with the MHC antigens of the H-2^d haplotype, whereas the *gag* protein under investigation by van der Hoorn and coworkers is recognised when presented with H-2^b. The fact that Flyer *et al.* (1985) report the lysis of their *env* expressing transfected cell line by syngeneic Tc also generated in H-2^d suggests that *env* antigens can be recognised when presented in association with MHC antigens of the H-2^d.

Major histocompatibility complex restricted recognition of MLV induced tumours

MHC H-2 restriction

As discussed earlier in this chapter the recognition of antigenic determinants on syngeneic cells by Tc is characterised by the phenomenon of H-2 restriction, that is, only those antigen-bearing target cells that display the same H-2 antigens as the effector T cells are lysed (reviewed by Schwartz, 1985). Many groups have shown this to be the case for target cells expressing Mo MLV antigens (Blank and Lilly, 1977; Plata and Lilly, 1979). Early work by Gomard and colleagues demonstrated that cytolysis of Moloney MLV lymphoma cells by MSV/MLV specific syngeneic T lymphocytes is restricted by some products of the H-2 complex (Gomard *et al.*, 1976). Further experiments mapped this restriction to the H-2D and H-2K regions of the MHC (Blank and Lilly, 1977). Furthermore, other early studies examining H-2 restriction showed that the involvement of H-2K and H-2D is not equivalent, in fact in most cases the products of only one region, H-2D or

H-2K, are involved in Tc recognition of the target cell (Gomard *et al.*, 1977). At the same time, similar phenomena were observed in other systems, with other viruses (Zinkernagel *et al.*, 1978; Doherty *et al.*, 1978; Pfizenmaier *et al.*, 1978), minor histocompatibility antigens (Wettstein, 1977) and hapten-modified cells (Levy and Shearer, 1979). Further evidence concerning this phenomenon will now be discussed.

MHC H-2 immunodominance

Within H-2 restriction, it appears that viral antigens are often recognised more efficiently when presented in association with certain MHC antigens rather than with others. For instance, congenic mice which differ in their H-2 MHC but are otherwise genetically identical generate Tc responses of different intensities to Gross MLV-induced syngeneic leukemias (Plata and Lilly, 1979). It was found that BALB.B ($H\text{-}2^b$) produced high numbers of Gross MLV-specific Tcm, BALB.K ($H\text{-}2^k$) mice generate very low numbers and BALB/c ($H\text{-}2^d$) mice produced an intermediate number of Tc. These Tc responses have been shown to reflect the resistance of these mice to syngeneic Gross MLV-induced leukemia cells. 'High leukemic' mouse strains, such as AKR, tend to be of the $H\text{-}2^k$ haplotype, whereas strains of $H\text{-}2^b$ haplotype are 'low leukemic' (Lilly, 1971; Green, 1966).

Early studies by Gomard *et al.* (1977) tested Tc derived from different inbred lines and their F1 hybrids or congenic partners against tumour cells of various H-2 specificities. They showed that in the mouse strains C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) Tc recognition of Mo MLV tumours is associated with only one class I antigen; D^b for C57BL/6 and K^d for BALB/c. There was no recognition observed in association with either K^b and D^d respectively. These and other observations (Gomard *et al.*, 1980) lead to

the proposal that there exists a hierarchy of H-2 plus viral antigenic association, with the best available association being immunodominant and thus precluding the recognition of the association of viral antigens with other class I antigens by Tc (Gomard *et al.*, 1980). This has been confirmed by recent studies carried out by other groups. For example, to identify the viral and H-2 antigens recognized by anti-Friend virus (FMV) Tc Holt and colleagues (1986) constructed a series of cell lines expressing putative target molecules, by DNA-mediated gene transfer. In their experiments they used a rat fibroblast line as the parental line to produce the target cells. It is argued that this approach allows an evaluation of the relative contribution of each transfected mouse gene to the generation of cell-surface target structures recognized by the murine cytotoxic T lymphocytes. Without interference from endogenous proteins and viral sequences they found that cells expressing the *env* or *gag* genes of FMV were susceptible to lysis by anti-FMV BALB.B (H-2^b) mice, whereas cells expressing H-2K^k instead of H-2K^b were resistant to lysis.

Stukart and coworkers (1982) examined the role of the H-2 D^b locus in the Tc response against Mo MLV with two strains of mice carrying a mutation in the H-2 D^b locus, B6.C-H-2^{bm13} (bm13) and B6.C-H-2^{bm14} (bm14). They reported that the influence of the D locus can be seen at two levels. First, neither of the two D^b mutant target cells could be recognised by D^b restricted Tc, as demonstrated with K^b D^b and K^b D^b effector cells. Secondly, mutation at the D^b locus strongly altered the character of the response, either by causing nonresponsiveness (bm14 effector) or by increasing the K^b associated component of the Tc response (bm13 effector). Two main theories have been proposed to explain T cell unresponsiveness to an antigen in association with a particular MHC molecule. First, absence of T cells in the repertoire due to negative selection during thymic development because

of a homology with self proteins (Barzofsky, 1987; Guillet *et al.*, 1987). Second, a defect at the level of antigen presentation, that is, a failure of antigen to enter into an immunogenic association with H-2 molecules (Sprent and Webb, 1987).

The observation that MHC antigens can bind peptide (as discussed in section 1.2) led to the suggestion that MHC molecules that are immunodominant in relation to a particular antigen may bind that antigen more efficiently than the other MHC molecules, which appear to be unresponsive. Experiments by Babbitt and colleagues (1985; 1986) examined this issue with the use of an equilibrium dialysis system in which 16 amino acid fragments of hen egg lysozyme (HEL) (Iwamoto *et al.*, 1986) were incubated with purified I-A^k molecules, previously shown to be a high responder, versus the low responder A-I^d molecule. It was found that the HEL peptide forms a strong association with the A-I^k molecules, whereas no measurable binding to the A-I^d molecules was observed. These results were confirmed by Buus *et al.* (1986b) who in addition showed that another low responder molecule, I-E^k, also failed to bind HEL. Similar experiments were carried out by Buus and coworkers (1986b) with another antigen, a peptide of ovalbumin. They found a quite different binding pattern but again it directly correlated with unresponsiveness, that is, binding to the high responder molecule I-A^k was observed but not to the low responders, I-A^b and I-E^k. Thus it is concluded that the ability of a specific antigen to bind a particular MHC molecule may determine which association is immunodominant.

Recent studies indicate that the processing of proteins such as HEL (Gammon *et al.*, 1987) and myoglobulin (Brett *et al.*, 1988) may also influence the hierarchy of determinants recognized by the expressed T cell repertoire. This is proposed as immunization with individual synthetic peptides of

these proteins has revealed additional T cell determinants not seen following immunization with the native molecule (Gammon *et al.*, 1987; Brett *et al.*, 1988). It is proposed by Brett *et al.* (1988) that the natural product of processing, which is larger than the synthetic peptide representing the minimal antigenic site, may have hindering structures that interefere with binding in the groove (Bjorkman, 1987a; Bjorkman, 1987b) of one MHC molecule but not another. This could occur even though the MHC binding portion of the antigenic site itself could bind equally well to the different MHC molecules. Furthermore, it has been suggested by other groups (for example Allen and Unanue, 1986) that MHC molecules may themselves guide processing by binding immunogenic peptides and protecting them from further degradation. Indeed, the groove of the proposed antigen binding site of MHC molecules (Bjorkman, 1987a; Bjorkman, 1987b) could provide a potential site for hindering further degradation.

Immune responses to *ras* transformed cells

Several groups have reported that fibroblasts transformed with the v-Ki-*ras* oncogene can be recognised and killed by natural killer (NK) cells and these cells may play an early role in host antitumour surveillance (Johnson *et al.*, 1985; Trimble, 1988). These results are consistent with the 'missing self' hypothesis, that NK cells can recognise and eliminate cells that fail to express self MHC class I molecules, suggested by Kärre *et al.* (1986), as it is known that *ras* expression down regulates constitutive expression and induction of MHC antigens (discussed in section 1.6). The *in vivo* studies reviewed by Ljunggren and Kärre (1990) that support this theory also can be reconciled with the earlier observations of MSV/MLV tumour regression. It has been found that only small not large tumour inocula can be rejected if the tumour cells are H-2 deficient as in the

case of MSV infected or v-*ras* transformed cells and the depletion of NK cells with monoclonal antibodies abrogates this response (Verateegh *et al.*, 1989). Thus it is proposed in the MSV/MLV tumour regression situation described earlier, the large inoculum of MSV transformed cells in preimmunized mice overwhelmed the limited nonadaptive NK cell response resulting in tumour growth, whereas the large inoculum of MSV/MLV transformed cells in preimmunized mice would be eliminated by the Tc adaptive response specific for the MLV antigens as previously discussed. It may also be possible that in the presence of the dominant immune response to MLV antigens, the responses to *ras* gene products are suppressed and thus mice immunized with MSV/MLV infected cells are unable to reject a tumour of MSV transformed cells.

More recently Chaever *et al.* (1990) and Peace *et al.* (1991) have shown that a T cell immune response to the mutated *ras* protein can be elicited by immunization with synthetic peptides constructed to be identical to the mutated portion of the *ras* protein. Peptides consisting of 12 or 13 amino acid residues which corresponded to the amino acid sequence from residue 5-16 or 5-17 of p21 were constructed to contain the normal glycine at the position corresponding to residue 12 (termed Gly-12) or alternatively the abberant substitution of arginine (termed Arg-12) or serine (Ser-12). Lymphocytes from C57BL/6 mice immunised with a single dose of the Arg-12 peptide were tested for a proliferative response to the above peptides *in vitro*. A proliferative response against the Arg-12 peptide but not the Gly-12 and Ser-12 peptides was found.

In conclusion although Tc responses against the MLV proteins are the main cause of tumour regression in the MSV/MLV system, an immune response can be elicited against the *ras* gene product p21. The importance of this response

in the regression of tumours has yet to be determined and is under investigation by fellow members of the CRC research group at Warwick University. One of the aims of this study is to further investigate the immune response elicited by the MLV proteins.

1.8 Retroviral vectors and selection systems

As discussed in detail before the main aim of this project was to generate tumour cell lines expressing well-defined antigens which could be used to study the importance of IFN modulation of T cell mediated immune responses to these antigens. The antigens used in this study were the *gag* and *env* polypeptides expressed by Ki and Mo MLV and the relative importance of these two viral antigens in the immune response to the MSV/MLV tumour is also examined. The approach taken by other groups to examine this response has been to construct cell lines that express the individual proteins in the absence of other potential MLV antigens. As mentioned in section 1.7, where the results from these groups are examined, one problem with the approach taken by these groups is that they use neomycin resistance to select for positively transfected cells. It is proposed in this study that the gene product of the neomycin resistance gene can itself elicit an immune response and evidence to support this theory is presented in section 4.7. One of the objectives of this project is to establish a procedure for selecting cells which have been successfully transfected with a gene of interest without introducing other neoantigens, such as antibiotic resistance selectable markers. Initially attempts were made to directly select the cells expressing the protein of interest using fluorescent labelled antibody and the fluorescence activated cell sorter (FACS). As discussed in section 3.4 this was found not to be possible, therefore an alternative method for selection was proposed. The procedure appears to be successful and it is hoped may be used in other studies.

The MHC class I D^b selection system

The selection system established in this study is described in the following text. The procedure was made possible by the relatively recent

availability of the cloned MHC class I gene D^k (Shar *et al.*, 1985) and the monoclonal antibody HB19 (supplied by D. J. Maudsley) which can be used to detect the H-$2D^k$ antigen. The cloned H-$2D^k$ gene is transfected into the H-$2D^b$ negative embryo fibroblast cell line C3H10T½ (H-2^k) with the DNA of interest, in this case the gene encoding the MLV protein(s). Cells expressing the H-$2D^k$ are sorted on the FACS after treatment with γ-IFN. To achieve a uniformly positive population of cells it is necessary to repeat the sorting procedure, the actual number of sorts necessary being dependent on the transfection and sorting efficiency. By ligating the DNA of interest to the H-$2D^k$ gene it is possible to ensure that all cells that take up the MHC gene also recieve the unselectable gene. The resulting cell line should be positive for both the MHC class I antigen D^k and the protein of interest. The parental mouse strain (C3H/He) of the transfected cell line C3H10T½ would recognise the MHC D^k antigen as foreign, therefore the experiments to determine the immunological importance of the antigen of interest are performed in hybrid mice, which do not recognise the D^k class I antigen as foreign. These mice are the F1 progeny of a cross between C3H/He (H-2^k) and C57BL6 (H-2^b) mice and thus they recognise both the endogenous and acquired MHC antigens of the transfected cell line as self. The proposal that this system can be used to examine the immune response to protein without actually interfering with the response they elicit is supported by the data in section 3.4.

The D^k selection system and the importance to tumourigenicity of point mutations in the *ras* oncogene

This selection system has been extended to another study undertaken in our group. Investigations are being carried out to determine the relationship between the point mutations at codon 13, 14, 59 and 61 of the *ras* oncogene and tumourgenicity. By site directed mutagenesis and subcloning, vectors

are being constructed that carry different permutations of the normal and viral codons at these four sites. When v-*ras* is transfected into nontransformed cells, the cells which take up and express the DNA can be selected for by their out growth in medium containing a lower percentage of serum than required for the growth of untransformed cells. Clearly by the nature of this study it is unknown whether the different *ras* constructs will transform the transfected cells therefore an alternative method for selection is required. By transfecting the mutated *ras* with the MHC D^b gene it is possible to select the positive cells as described above and the tumourgenicity studies can be carried out in the F1 hybrid mice. In this way it should be possible to determine the importance of the mutations at the four codons in relation to tumourgenicity. The progress made so far with this study is discussed later.

Retroviral vectors

The vectors constructed in this study use retroviral elements to express the viral proteins of interest. The proteins to be expressed in this work are retroviral proteins, however retroviral vectors have been used to express a whole range of exogenous genes in eukaryotic cells and there use has been well documented (Capko *et al.*, 1984; McIvor *et al.*, 1987; Zwiebel *et al.*, 1989; Miller and Rosman, 1989). An understanding of the integration stage of replication would be beneficial to those researchers designing retroviral vectors, however as discussed in section 1.6, the mechanism of integration has still to be made clear. Nonetheless, it does appear that linear molecules of DNA are the favoured form for integration and that the terminal inverted repeats of the LTR are involved, and thus are an important component of any retroviral vector. Additionally, it is known that virion protein, integrase, is also necessary for specific retrovirus integration. However, it is possible for LTR containing DNA to be

introduced stably into cells by transfection, although the efficiency and specificity typical of retroviral infection is lost (Weiss *et al.*, 1984).

A description of the construction of the vectors used in this work can be found elsewhere (section 3.1), however the salient points will be discussed here. The preliminary vector was constructed from a MLV clone by inserting the 5' and 3' LTR into the cloning vector pUC13. This vector, which is easily manipulated in *E. coli*, can be used to express cloned fragments inserted between the two LTRs. As described in section 1.6 retroviral LTRs contain the necessary transcriptional signals for expression of genes in most tissue cultures with the exception of cells of early developmental stages, such as undifferentiated embryonal cells (Feuer *et al.*, 1989). Therefore, it is not necessary for the inserted fragment to have its own transcriptional factors, as is the case for other vectors (Shimotohno and Temin, 1981; Wei *et al.*, 1981; Tabin *et al.*, 1982) as the 5' LTR supplies the transcription initiation signals etc. for RNA polymerase 2 and the 3' LTR provides the information for polyadenylation of the RNA transcripts. It has been shown conclusively that the LTR acts as a strong promotor/enhancer which greatly increases transcriptional efficiency (Chang *et al.*, 1980; Kriegler and Botchan, 1983; Khoury and Gruss, 1983). The U_3 region of the LTR has been reported to be involved in disease specificity (Stocking *et al.*, 1986) - possibly it is the enhancer sequences found in the U_3 region which are responsible for this specificity (Yoshimura *et al*, 1985). Gunther *et al.* (1990) reports that the proto-oncogene *ets*-1, which is differentially expressed in the thymus and spleen of adult animals, is specifically bound by the LTR of Mo MSV. They suggest that the *ets*-1 gene product functions as a transcription factor during thymocyte development and/or mature T-lymphocyte function. As the regions of the MSV LTR bearing the *ets*-1 binding site is highly conserved in the LTRs of all murine,

simian, and feline type-C retroviruses, it is proposed by Gunther *et al* (1990) that the ability of retroviral LTR to exploit this lymphoid transcription factor may explain in part the ability of Mo MLV to cause lymphomas by its successful replication in lymphoid tissue (Evans and Morrey, 1987; Speck *et al.*, 1990). CAAT and TATAA boxes sequences associated with RNA polymerase 2 promotor regions are also found in the LTR. Thus if a fragment which contains the coding sequence for a protein and a start codon for translation is inserted in the correct orientation between the two LTRs, the protein will be expressed when the vector is transfected into a tissue culture cell (with only a few exceptions).

Retroviral packaging cell lines

In recent years the use of retroviral vectors has become increasingly wide spread and this is mainly due to the availibility of retrovirus packaging cell lines which allow production of replication-defective retrovirus vectors in the absence of helper virus (Mann *et al.*, 1983; Miller and Buttimore, 1986; Markowitz *et al.*, 1988; Miller and Rosman, 1989; Joshi *et al.*, 1990). Such vectors are reported to infect and integrate into cells with great efficiency but cannot replicate and spread. These properties make possible a variety of studies in which virus spread would make interpretation of results difficult or impossible. In the first generation of helper-free packaging cell lines, expression of the necessary viral proteins was achieved through the stable introduction of a mutant Mo MLV proviral genome containing a 350 bp deletion of the ψ packaging signal, a sequence required for efficient encapsidation of the Mo MLV genome (Mann *et al.*, 1983). Thus the mutant genome has a defect in the packaging of genomic RNA into virions but can provide in *trans* the products necessary for the virion production. When replication-defective vectors carrying the packaging signal are transfected into this cell line (ψ-2), the ψ^- mutant

genome can enable the packaging of the ψ^+ RNA of the vector without being packaged itself. Although this cell line, and others similar, have been successfully used by many investigators (Mann *et al.*, 1983), it has been shown by some workers that virus-producing cell lines derived from ψ-2 produce low levels of virus containing the ψ^- genome and therefore are able to transfer the mutant proviral genome to recipient cells, at a low efficiency (Mann and Baltimore, 1985). Additionally, in a few cases, the encapsidation of the ψ^- genome appears to lead to the generation of wild-type virus through recombinational events involving a copackaged recombinant genome carrying the ψ sequence (Bosselman *et al.*, 1987; Bender *et al.*, 1987).

More recently, improvements have been made in these retrovirus packaging cells to reduce the potential of these cell lines to produce replication-competent helper virus while allowing the production of retroviral vectors at high titre (Miller and Buttimore, 1986; Danos and Mulligan, 1988; Markowitz *et al.*, 1988; Miller and Rosman, 1989). In addition, the recent discovery that the signal for packaging of viral RNA extends into the *gag* region of the virus has resulted in the construction of retroviral vectors including this region (Armentano *et al.*, 1987; Bender *et al*, 1987; Adam and Miller, 1988). It is reported that the inclusion of this region in vectors results in a 10-fold increase in the vector titre. However, it is necessary to ensure that additional proteins are not synthesized from this region and helper virus are not yielded by recombination between homologous overlap of *gag* sequences in the retroviral vector and sequences present in the the packaging cell line. These problems were overcome by Miller and Rosman when they designed the LNL6 vector. They inserted a stop codon in place of the $Pr65^{gag}$ start codon to prevent synthesis of $Pr65^{gag}$ and replaced the upstream region of the vector with the homologous region from Mo MSV which

does not make gPr85^{env}, thus preventing synthesis of viral proteins from this vector. To prevent the helper virus being generated by homologous recombination, all *env* sequences were removed from the vector and the defective helper virus used to produce the packaging cell line was truncated immediately after *env*, thus making it impossible to generate helper virus. The retroviral vectors constructed for the purpose of this study contain the packaging signal, thus if the vector was transfected into a packaging cell line, the RNA would be packaged into virion. During this study, packaging cell lines are not utilised, however the inclusion of this signal enables them to be used in the future, for instance, with primary cell lines which are more difficult to transfect by the calcium-phosphate method described in this study (2.9F) (R. Darley, personal communication).

The major aims of this work can be summarised as follows:

1. To generate tumour cell lines which express well-defined antigens. This will be achieved by expressing the individual MLV antigens, *gag* and *env*, in the *ras* transformed fibroblast cell line C3H201.
2. To use these cells to study the importance of interferon modulation of T cell mediated immune responses to these cells.
3. To examine the relative importance of the *gag* and *env* antigens in the tumour regression of cells infected with MSV/MLV.
4. To use a molecular clone of the MLV genome lacking the ψ packaging signal to examine the response to the MLV antigens together, without the complications associated with the presence of infectious virus.
5. To use the MHC D^{b} antigen to develop a selection system that does not itself alter the immunological properties of the transfected cells.

Materials

2.1 Vectors

The following vectors were used during this study:

Name	source	Reference
pAT153 KCC7	J. Horton	Horton *et al.*, 1982
pAT153 cP2	J. Horton	Horton *et al.*, 1984
pBR327 D^{-}	A. Mellor	Weiss *et al.*, 1984
pGEM-1	Promega Biotec	Melton, D. A. *et al.*, 1984
pMOVψ^{-}	R. Mulligan	Mann *et al.*, 1983
pNEO	Pharmacia	Pharmacia catalogue
pSV2-neo	G. Ward Warwick University	Southern and Berg, 1982
pUC13	G. Ward	Yanisch-Perron *et al.*, 1985
pUC LTR KCC7	G. Ward	Personal communication
pUC13 Mo LTR	G. Ward	Personal communication
pZAP	A. Evans	Personal communication

2.2 Bacterial strain

The *E. coli* K12 strain TG2 was used for all recombinant DNA manipulation. It is derived from strain JM101 (Messing *et al.* 1981) and its genotype is as follows:

Δ(*lac-pro*), *thi*, *strA*, *hsdR*$^{-}$, *recA*$^{-}$,
sc1C 300:Tn10(tet)/F' traD36, *proAB*, *lacIq*, *lacZ-M15*.

TG2 was grown on LB media but was stored at 4°C on solid minimal salts medium or stored at -70°C, as described by Maniatis *et al.* (1982). All manipulations of TG2 were carried out using standard aseptic techniques.

2.3 Cell lines

All cell lines used were stored long-term in liquid nitrogen and when required were thawed and maintained in continuous culture for at least 10 days before use.

Cell type	Description	Source	Reference
C3H10T½ clone 8	embryo fibroblast line cloned from C3H mouse	American Type Culture Collection	Reznikoff *et al.*, 1973
C3H201	C3H10T½ infected with helper-free Ki-MSV	A. G. Morris Warwick University	Maudsley and Morris, 1988
KC3H	C3H10T½ infected with the complex Ki-MSV/MLV	A. G. Morris Warwick University	Maudsley and Morris, 1988
KLVC3H	C3H10T½ infected with Ki-MLV	A. G. Morris Warwick University	Maudsley and Morris, 1988
MoSVC3H	C3H10T½ infected with the complex Mo-MSV/MLV	D. J. Maudsley Warwick University	
WR 19L	T cell lymphoma infected with Mo-MLV	W. J. Bateman Warwick University	Raschke *et al.*, 1978
YAC-1	Mo-MLV induced lymphoma line from A/Sn mouse	American type Culture Collection	Kiessling *et al.*, 1975

All cells, except WR 19L and YAC-1, were cultured in GMEM complete. WR 19L and YAC-1 were cultured in RPMI complete. All manipulations of the above cell lines were carried out under sterile conditions using standard aseptic techiques.

2.4 Mice

Male and female C3H/He ($H\text{-}2^{a}$) mice were obtained from a breeding colony present within this department, which was originally established using breeding pairs supplied by Bantin and Kingman. The F1 progeny from a cross between female C57 BL/6 ($H\text{-}2^{b}$) and C3H/He male ($H\text{-}2^{a}$) mice were supplied by Olac ltd. or by the departmental animal house. The C57 BL/6 mice used by the animal house for this cross were originally supplied by Bantin and Kingman.

2.5 Media and Antibodies

All media used were sterile.

A) Bacterial growth media

Luria-Bertani (LB) medium

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g
distilled water	to 1 litre

Autoclave at 121°C for 15 minutes

LB agar selection plates

bacto-agar	6 g
LB medium	300 ml
ampicillin 100 mg/ml stock (filter sterilised)	300 µl
isopropyl-B-thiogalactopyranoside (IPTG) 100 mM stock (filter sterilised)	150 µl
5-bromo-4-chloro-3-indolyl-B-galactosidase (X-gal) 2% (w/v) stock (filter sterilised)	500 µl

5x minimal salts

K_2HPO_4	5.25 g
KH_2PO_4	2.25 g
$(NH_4)_2SO_4$	0.5 g
trisodium citrate.$2H_2O$	0.25 g
distilled water	100 ml

Autoclave at 121°C for 15 minutes

Minimal salts medium

5x minimal salts	100 ml
20% (w/v) glucose (filter sterilised)	10 ml
1 M $MgSO_4$ (autoclaved)	0.5 ml
1% (w/v) thiamine HCl (filter sterilised)	125 µl
sterile distilled water	to 500 ml

2YT

bacto-tryptone	16 g
bacto-yeast extract	10 g
NaCl	10 g
distilled water	to 1 litre

Autoclave at 121°C for 15 minutes

B) Tissue culture media

The Glasgow modification of Eagle's minimum essential medium (GMEM) buffered with sodium hydrogen carbonate (pH 7.0), Roswell Park Memorial Institute (RPMI) 1640 buffered with sodium hydrogen carbonate (pH 7.0), double strength GMEM minus methionine, valine, leucine, glutamine and sodium hydrogen carbonate, distilled water, antibiotics (penicillin and streptomycin), phosphate buffered saline (PBS), non-essential amino acids (NEAA), 2 M Hapes, versene and 1% trypsin in PBS were prepared as standard stock solutions and sterilised by B. Wood (University of Warwick). Unbuffered RPMI 1640 was commercially supplied.

Foetal calf serum (FCS) was heat-inactivated for 45 minutes at 56°C in order to inactivate complement prior to use.

GMEM complete

GMEM pH 7.0	1x
FCS	10% (v/v)
glutamine	4 mM
penicillin	60 µg/ml
streptomycin	100 µg/ml.

Interferon-γ

Murine recombinant IFN-γ was prepared in this laboratory from Chinese hamster ovary cells transfected with the mammalian expression vector pKSV10 containing a cDNA copy of the IFN-γ mRNA (Morris and Ward, 1987). The IFN

present in supernatants harvested from the transfected cells was then partially purified by affinity chromatography on cibacron blue sepharose and titrated by S. McQuiston. Preparations of IFN-γ were filter sterilised before use.

Methionine-free medium

2x GMEM lacking glutamine, leucine, valine, methionine	1x with DW
glutamine	4 mM
leucine	2 mM
valine	2 mM
penicillin	60 µg/ml
streptomycin	100 µg/ml

Mixed lymphocyte tumour cell culture (MLTC) medium

RPMI 1640	1x
FCS	10% (v/v)
glutamine	8 mM
ß-mercaptoethanol	10^{-5} M
penicillin	60 µg/ml
streptomycin	100 µg/ml
NEAA	1x
Hepes	12 mM

10x NEAA

L-alanine	0.89 g
L-asparagine.H_2O	1.50 g
L-aspartic acid	1.33 g
glycine	0.75 g
L-glutamic acid	1.45 g
L-proline	1.15 g
L-serine	1.05 g
distilled water to	1 litre

RPMI complete

RPMI pH 7.0	1x
FCS	10% (v/v)
glutamine	4 mM
penicillin	60 µg/ml

streptomycin	100 μg/ml

Trypsin/versene

trypsin solution (1% stock)	20% (v/v)
versene	80% (v/v)

C. Antibodies

1. Murine monoclonal anti-H-2D$^{b/d}$ was provided by D. J. Maudsley (University of Warwick) as ascites fluid produced from a hybridoma clone 28-11-5S (ATCC HB19).

2. Murine monoclonal anti-H-2K^{k} was provided by D. J. Maudsley (University of Warwick) as ascites fluid produced from a hybridoma clone 11-4-1 (ATCC TIB95).

3. Goat polyclonal serum anti-p30 (Ki and Ho) were purchased from The National Cancer Institute, Bethesda, U. S. A.

4. Goat polyclonal serum anti-gp70 (Ki and Ho) were purchased from The National Cancer Institute, Bethesda, U. S. A.

5. Fluorescein isothiocyanate (FITC) conjugated goat-anti-mouse and rabbit-anti-goat IgG fractions were obtained from Cappel.

2.6 Solutions and buffers

All solutions made up in distilled water unless otherwise stated.

40% (w/v) Acrylamide stock

acrylamide	39.2% (w/v)
N, N-methylenebisacrylamide	0.8% (w/v)

Aqueous hybridisation solution

Denhardt's solution	5x
SSC	6x
denatured salmon sperm DNA	100 µg/ml

Bio-Rad HRP developing solution

prepare and mix solution A and B just prior to use

Solution A	
Tris buffered saline	100 ml
hydrogen peroxide 100 volumes	60 µl
Solution B	
HRP colour development reagent (4-chloro-1-napthol)	60 mg
methanol	20 ml

10x CIAP buffer

Tris-HCl pH 9.0	0.5 M
$MgCl_2$	10 mM
$ZnCl_2$	1 mM
spermidine	10 mM

50x Denhardt's solution

Ficoll	5 g
BSA	5 g
polyvinylpyrrolidone	5 g
distilled water	to 500 ml

Diaminobenzidine (DAB) developing solution

DAB	10 mg
PBS	20 ml
hydrogen peroxide 100 vols (added just before use)	400 µl

Gel fixing solution

methanol	250 ml
glacial acetic acid	35 ml
distilled water	to 500 ml

Isotonic lysis buffer

NaCl	150 mM
$MgCl_2$	1.5 mM
Tris-HCl pH 7.8	10 mM

Nonidet P-40	0.65% (v/v)

10x ligation buffer

Tris-HCl pH 7.4	0.5 M
$MgCl_2$	0.1 M
dithiothreitol	0.1 M
spermidine	10 mM
ATP	10 mM
BSA	1 mg/ml

Lysis buffer

Nonidet P-40	2 ml
PBS	98 ml

10x nick translation buffer

Tris-HCl pH 7.2	0.5 M
$MgSO_4$	0.1 M
dithiothreitol	1 mM
BSA	500 µg/ml

5x OLB (oligo labelling buffer)

5x OLB consists of solutions A, B and C

in the ratio of 10:25:15

5x OLB solution A

solution C	1 ml
14.3 M β-mercaptoethanol	18 µl
100 mM dATP in 3 mM Tris-HCl pH7.0, 0.2 mM EDTA	5 µl
100 mM dTTP in 3 mM Tris-HCl pH7.0, 0.2 mM EDTA	5 µl
100 mM dGTP in 3 mM Tris-HCl pH7.0, 0.2 mM EDTA	5 µl

stored at -20°C

5x OLB solution B

Hepes pH 6.6	2 M

stored at 4°C

5x OLB solution C

pd(N)$_6$ Pharmacia random primers	
50 OD units in 550 µl $T_{10}E_1$ to give	9000/ml

5x OLB solution O

Tris-HCL pH 8.0	1.25 M
$MgCl_2$	0.125 M
store at 4°C	

Phosphate buffered saline (PBS)

potassium chloride	0.2 g
sodium chloride	8.18 g
potassium dihydrogen orthophosphate	0.2 g
di-sodium hydrogen orthophosphate	1.15 g
distilled water	to 1 litre

Phenol

The phenol stock consisted of distilled phenol (Wallace, 1987), equilibrated by shaking with equal volume of phenol equilibration buffer with several changes. It was stored under phenol equilibration buffer at 4°C.

Phenol/chloroform

Equal volumes of phenol stock and chloroform were mixed together and stored under phenol equilibration buffer at 4°C.

Phenol equilibration buffer

Tris-HCl pH 7.5	10 mM

NaCl	300 mM
EDTA	1 mM

Phenol extraction buffer

Tris-HCl pH 7.8	10 mM
NaCl	350 mM
EDTA	10 mM
SDS	1% (w/v)
urea	7 M

Plasmid preparation solution I

glucose	50 mM
Tris-HCl pH 8.0	25 mM
EDTA pH 8.0	10 mM
lysozyme (added just before use)	5 mg/ml

Plasmid preparation solution II

NaOH	0.2 N
SDS	1%

Plasmid preparation solution III

potassium acetate	5 M
glacial acetic acid to pH 4.8	

Ponceau S

Ponceau S	0.5% (w/v)
glacial acetic acid	2% (v/v)

Resolving gel (10%)

40% (w/v) acrylamide stock	12.5 ml
3.0 M Tris-HCL pH 8.8	6.7 ml
10% (w/v) SDS	0.5 ml
distilled water	29.8 ml
10% (w/v) ammonium persulphate (added just before use)	0.5 ml
TEMED (added just before use)	20 µl

Radioimmunoprecipitation (RIP) lysis buffer

Nonidet P-40	2 ml
PBS	98 ml
PMSF (added just before use)	1 mM

RIP wash buffer

LiCl	500 mM
Tris-HCl pH8.5	100 mM

SDS/PAGE running buffer

glycine	29.9 g
Trizma base	6.0 g
SDS	2.0 g
distilled water	to 2 litre

3x SDS/PAGE sample buffer

glycerol	3 ml
14.3 M β-mercaptoethanol	1.5 ml
1 M Tris-HCl pH 6.8	1.5 ml
SDS	0.6 g
15% (w/v) bromophenol blue	1 ml
distilled water	3 ml

20x SSC

NaCl	175.3 g
sodium citrate	88.2 g
distilled water	to 1 litre

Stacking gel (6%)

40% (w/v) acrylamide stock	3 ml
0.5 M Tris-HCl pH 6.8	5 ml
10% (w/v) SDS	0.2 ml
distilled water	11.8 ml
10% (w/v) ammonium persulphate (added just before use)	100 µl
TEMED (added just before use)	15 µl

5x TBE loading buffer

TBE	5x
bromophenol blue	0.25% (w/v)
glycerol	25% (v/v)

50x TAE

Trizma base	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
distilled water	to 1 litre

10x TBE

Trizma base	54 g
boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
distilled water	to 500 ml

$T_{10}E_1$

Tris-HCl pH 8.0	10 mM
EDTA pH 8.0	1 mM

1 M Tris buffer

Trizma base	121.1 g
distilled water	to 900 ml
hydrochloric acid to the required pH and	
then to 1 litre with distilled water	

Tris buffered saline

Tris-HCl pH 7.5	20 mM
NaCl pH 7.5	500 mM

Western blot transfer buffer

Trizma base	12.12 g
glycine	57.6 g
Methanol	800 ml
distilled water	to 4 litre

2.7 Names and addresses of suppliers

All chemical and reagents except those listed below were obtained from BDH Chemicals Ltd., Poole, Dorset, U. K. and were of AnalaR grade.

Alcan Ltd., 90 Ashbridge Road, Chesham, Buckinghamshire, U. K.

Aluminium foil

Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, U. K.

Amplify
Anti-goat immunoglobulin biotinylated antibody
α-^{35}S dATP
α-^{35}S methionine
^{51}Cr (sodium chromate)
restriction enzyme
Streptavidin biotin horse radish peroxidase complex

Bantin and Kingman, The Field Station, Grimston, Aldbrough, U. K.

C3H/HE mice
C57 BL/6

Beckman RIIC Ltd., Progress Road, High Wycombe, Buckinghamshire, U. K.

Calibrite beads
Ready-solv EP liquid scintillation fluid
Tissue culture flasks
Tissue culture petri-dishes 6 cm

BRL (Bethesda Research Laboratories) Ltd., Renfrew Road, Paisley, U. K.

BLue GENE nonradioactive nucleic acid detection system
3'3' diamino benzidine. HRP colour development reagent

Immuno-precipitin
1 Kb ladder
Restriction enzymes

Cappel, West Chester, PA, 19380, U. S. A.

FITC conjugated antibodies

Difco Laboratories, Detroit, U. S. A.

Bacto-agar
Bacto-tryptone

Fisons plc (Services), Crawley, Sussex, U. K.

Acrylamide
Butan-1-ol
Chloroform
Dialysis tubing
Diethyl ether
Dimethyl sulphoxide
Hyrodrogen peroxide
Trichloroacetic acid
Urea

Flow Laboratories Ltd., Woodcock Hill, Rickhamsworth, Hertfordshire, U. K.

Flow pore 0.45 µm filter sterilizing units
Freezing ampoules 2 ml
Glutamine
Microtitre 96 well trays round-bottomed
Microtitre 96 well tray adhesive sealers

Fuji Photo Film Co. (UK) Ltd., 125, Finchley Road, London, U. K.

RX medical X-ray film

Gibco Europe Ltd., Trident House, Paisley, Scotland.

Dulbecco's modification of Eagles minimum essential medium
Foetal calf serum
Glasgow modification of Eagles minimum essential medium
Roswell Park Memorial Institute (RPMI) 1640

Glaxo Laboratories Ltd., Greenford Road, Greenford, Essex, U. K.

Penicillin
Streptomycin

Kodak Chemical Ltd., Kirby, Liverpool, U. K.

N'N-methylene bis-acrylamide
Photographic paper, Kodabromo II RC

L. I. F. Equipment and Services, 111, Dockfield Road, Shipley, Yorkshire, U. K.

Micropipette tips, 200 µl and 1 ml

May and Baker Ltd., Liverpool Road, Eccles, Manchester, U. K.

Acetic acid (glacial)
Ethanol
Hydrochloric acid
Methanol

Nippon Shaji Kaisha Ltd., Osaka, Japan

Neocofilm

Northumbria Biologicals Reagents Ltd., South Nelson Industrial Estate, Cramlington, Northumberland, U. K.

Foetal calf serum

Nycomed Ltd., 11 Wagon Lane, Sheldon, Birmingham, U. K.

Lymphoprep

Olac ltd., Shaws Farm, Bicester, Oxon, U. K.

C3H BL/6 FI progeny

Pharmacia LKB Biotechnology Ltd., Midsummer Boulevard, Milton Keynes, U. K.

Electrophoresis calibration kit, molecular weight markers
Deoxynucleotides
Klenow fragment of DNA polymerase I
pMEO vector
Scintillation tubes 6 ml

Promega Biotec, 2800 S Fish Hatchery Road, Madison, U. S. A.

pGEM-1 vector

Sabre International, Manor Farm Road, Reading, U. K.

Hypordermic needles 21, 25 and 26⅝ gauge

Sarstedt Ltd., 68, Boston Road, Beaumont Leys, Leicester, U. K.

Eppendorf tubes 0.75 and 1.5 ml

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, U. K.

Agarose type II medium EEO
Bovine serum albumin
Cibacron blue sepharose
Crystal violet
Ethidium bromide
Lysozyme
Mitomycin C
Non-essential amino-acids
PMSF
Ribonuclease A
Tween 20
Ponceau S
Triton X 100

Sterilin Ltd., 43, Broad Street, Teddington, Middlesex, U. K.

Plastic bijoux
Plastic test-tube, 4 mm
Plastic universal
Syringes: 1, 2, 5, 10, 20 and 50 ml

Whatman Ltd., Unit 1, Coldred Road, Parkwood, Maidstone, U. K.

Glass wool
Whatman filter paper

2.8 DNA manipulations

A) Phenol extraction

Phenol extractions were carried out to remove enzymes and other contaminating proteins from nucleic acid samples. An equal volume of phenol was added to the sample, it was vortexed for 10-30 seconds and then centrifuged for 2-10 minutes depending upon the volume involved. The upper aqueous phase was removed to a fresh tube and re-extracted with phenol until the interface was clear. The samples were then extracted with an equal volume of chloroform and the nucleic acid was ethanol precipitated. Phenol/chloroform extractions were carried out in the same way using phenol/chloroform instead of phenol.

B) Ethanol precipitation

DNA was ethanol precipitated when necessary to concentrate the DNA, remove traces of organic solvents or resuspend the DNA in a different buffer. The DNA solution was made 300mM with respect to sodium chloride, using a stock of 3 M sodium chloride (pH 7.0). Then 2 volumes of ice cold absolute ethanol were added, the sample was vortexed and it was incubated either in a dry ice/ethanol bath for 10-30 minutes, -70°C for 1-3 hours or -20°C overnight. The sample was then centrifuged, conditions depending on the volume of the sample, washed in 70% (v/v) ethanol and dried in a vacuum dessicator.

C) DNA restriction digests

DNA was digested with a 4-5 fold excess of restriction enzyme units, under the conditions recommended by the supplier. The restriction enzyme added was not more than one tenth of the total volume of the reaction. When DNA was cleaved with a combination of enzymes, digestions were performed sequentially, adjusting reaction conditions appropriately for the following enzyme(s). The reactions were stopped either by the addition of 5x TBE loading buffer in the case of samples to be analysed by agarose gel electrophoresis or by phenol/chloroform extraction for samples that were to be used for further manipulations.

D) Gel electrophoresis of DNA

Agarose gel electrophoresis was used to resolve products of restriction enzyme digestions and also to isolate specific DNA fragments by means of fragment preparations. The agarose concentration within the gel varied between 0.5% and 2.0% (w/v) depending on the size of DNA molecules to be resolved, with 1% most commonly used. The relationship between agarose concentration and the resolving characteristics of a gel is described by Maniatis *et al.* (1982). Two types of gel apparatus were used. The Pharmacia minigel apparatus was used for small quantities of DNA (10-20 ng), for rapid analysis of restriction enzyme digest products or DNA quantification and the BRL large horizontal gel system was used for larger quantities of DNA or for high resolution of the DNA bands. The methods are essentially the same for the two systems with the exception of the volume of agarose used, 25 ml for the minigel and 250 ml for the larger gel. The appropriate weight of agarose was dissolved in 1x TBE (or 1x TAE), the agarose solution

was poured into a gel former with a comb forming the wells. The type of comb used was dependant on the sample size and the number of samples to be run on the gel. The gel was left to set for 30 minutes and then was placed in the gel tank and submerged in 1x TBE (or 0.3x TAE) containing 0.5µg/ml of ethidium bromide. DNA samples were prepared by adding one fifth the volume of 5x TBE (or TAE) loading buffer and were loaded into the wells. The gels were electrophoresed at 100-150 V for 1-2 hours or 30 V overnight. The DNA was visualised by placing the gel on a UV light box and photographed using a Polaroid camera and Polaroid Type 55 4 x 5 land film.

E) DNA modifications

1) Dephosphorylation of DNA

The removal of terminal 5' phosphate groups from linear DNA with calf intestinal alkaline phosphatase (CIAP) was achieved as described in Maniatis *et al.* (1982). The reaction contained up to 1 µg of linearised DNA, 0.1 volume of 10x CIAP buffer and six units of CIAP enzyme. The reaction was stopped by phenol/chloroform extraction and ethanol precipitation of the DNA.

2) DNA ligation

The method for high efficiency ligation of sticky or blunt DNA ends was modified from Rusche and Howard-Flanders (1985). Ligation was carried out in 10-25 µl reaction containing 50-100 ng of linearised vector with varying amounts of insert, 0.1 volume of BRL 10x ligation buffer and 10 units of T4 DNA ligase. The reaction was incubated overnight at 15°C and the ligation mix was diluted 1 in 5 before use in a transformation.

3) Blunt ending of DNA

The ends were blunted using either the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase as described in Maniatis *et al.* (1982). The reaction was stopped by the addition of EDTA and the DNA was phenol/chloroform extracted and ethanol precipitated.

F) DNA Transformation of *E. coli*

1) Preparation of competent cells

1 ml of an overnight culture of *E. coli* TG2 was subcultured into 50 ml of LB medium and incubated at 37°C in an orbital shaker (200 rpm) until the optical density of the culture was approximately 0.4 at 590 nm. The cells were left on ice for 30 minutes and then transferred to two 25 ml universal tubes and pelleted by centrifugation (4 000 rpm, 5 minutes, 4°C). The pellets were resuspended in 20 ml of chilled freshly prepared 100 mM magnesium chloride. The cells were immediately centrifuged again and the pellet was resuspended in a total of 2.5 ml chilled freshly prepared 100 mM calcium chloride. The cells were left on ice for at least 1 hour before use.

2) Transformation of plasmid DNA

A 100 µl aliquot of competent cells was added to a 1.5 ml Eppendorf tube containing the DNA (1-25 µl) to be transformed and the cells were incubated on ice for 30 minutes. The cells were heat shocked by incubating at 42°C for 2 minutes then immediately quenched on ice for 5 minutes. After this heat shock period, 900 µl of LB medium was added and the cells were incubated at 37°C for 60 minutes. After the recovery period, varying amounts of the cells were plated onto LB agar plates containing 100 µg/ml

ampicillin, 50 μM IPTG and 0.002% (w/v) X-gal (Ruther, 1980) and incubated overnight at 37°C.

G) Preparation of plasmid DNA

1) Small scale plasmid preparation

5 ml aliquots of LB medium containing 100 μg/ml ampicillin were inoculated with ampicillin resistant colonies and the cultures grown overnight at 37°C in an orbital shaker (200 rpm). The bacteria were pelleted by centrifuging for 60 seconds in a Eppendorf microfuge and plasmid DNA was prepared by a method based on the alkaline lysis method of Birnboim and Doly (1979). Each bacterial pellet was resuspended in 100 μl of plasmid preparation solution I. After 5 minutes at room temperature 200 μl of plasmid preparation solution II was added and the contents were mixed by inverting the tubes several times. After incubation on ice for 5 minutes 150 μl of ice cold plasmid preparation solution III were added. The samples were vortexed, incubated on ice for 5 minutes then centrifuged in an Eppendorf microfuge for 5 minutes. The supernatants were transferred to fresh tubes, extracted with phenol/chloroform and the DNA ethanol precipitated from the aqueous layers. The washed and dried pellets were resuspended in 50 μl $T_{10}E_1$ containing 20 μg/ml of DNase-free pancreatic RNase and stored at -20°C. The DNA was analysed by digesting an aliquot (typically 5 μl) with the appropriate
restriction enzymes.

2) Large scale plasmid preparation

The method used was a modification of that described by Maniatis *et al.* (1982). 1 ml from an overnight culture was used to inoculate 25 ml of LB medium containing 100 μg/ml of ampicillin and incubated in an orbital

shaker (200 rpm) at 37°C until the A_{650} was 0.3. The culture was then added to 500 ml of LB medium containing 100 μg/ml of ampicillin and the incubation continued for 6 hours. After this time chloramphenicol was added to a final concentration of 100 μg/ml and the incubation continued overnight.

The plasmid DNA was prepared by a method based on the alkaline lysis method of Birnboim and Doly (1979). The bacteria was pelleted in a MSE 6L centrifuge at 2 500 rpm, 4°C for 30 minutes in a 6 x 1000 ml angle rotor and the supernatant was discarded. The pellet was resuspended in 8 ml of plasmid preparation solution I, transferred to a polypropylene Oakridge tube and incubated at room temperature for 5 minutes. 16 ml of freshly prepared plasmid preparation solution II was added, the contents mixed by inversion and incubated on ice for 10 minutes. 12 ml of ice cold plasmid preparation solution III was added, the solution was mixed by inversion and returned to ice for 10 minutes before centrifugation at 15 000 rpm in an 8 x 50 ml rotor of an MSE MS18 centrifuge for 20 minutes at 4°C. The supernatant was divided between two 30 ml Corex tubes, 0.6 volumes of isopropanol added and the DNA precipitated at room temperature for 15 minutes. The nucleic acid was recovered by centrifugation as before for 30 minutes at room temperature, the pellets were washed in 70% (v/v) ethanol and dried in a vacuum dessicator. Each pellet was resuspended in 5 ml of $T_{10}E_1$ and then combined together. The DNA was purified by banding on a caesium chloride density gradient.

3) Caesium chloride density gradient

This was based on the method described by Maniatis *et al.* (1982). 26 g of caesium chloride was dissolved in 16 ml sterile distilled water and was added to the 10 ml of nucleic acid solution with 0.2 ml of a 10 mg/ml stock of ethidium bromide. This mixture was loaded into a Beckman 38 ml Quickseal tube, liquid paraffin was added and the tube was sealed following the manufacturer's instructions. The tube and balance were centrifuged for 16 hours at 45 000 rpm and 15°C in a Beckman Vti50 rotor and L8 centrifuge. The DNA was visualised with long wave UV light and recovered by side puncture using a syringe. Ethidium bromide was removed by 5 extractions with isoamylalcohol and the the aqueous phase dialysed against 3 changes (each of at least 1 hour) of 5 litres of $T_{10}E_1$. The DNA was ethanol precipitated and resuspended in 1 ml of sterile distilled water and the concentration was estimated by measuring the OD of the solution at 260 nm (1 OD_{260} unit was assumed to be equivalent to 50 µg/ml DNA).

E) DNA fragment preparation

DNA fragments were recovered from agarose gels by one of the following methods.

1) Freeze-squeeze method

This method is based on the procedure described by Thuring *et al.* (1975). The DNA was electrophoresed and visualised on a UV light box as described before. The relevant band was cut from the gel using a scalpel, chopped into small pieces and placed into a 1.5 ml Eppendorf tube. The DNA-containing liquor was extracted from the agarose by repeated freezing in a dry ice/ethanol bath and thawing in a 37°C water bath. 200 µl of distilled

liquor was transferred to a fresh Eppendorf tube, phenol extracted 3 times, phenol/chloroform extracted once and ethanol precipitated. The DNA was resuspended in an appropriate amount of distilled water.

2) Electroelution into a dialysis bag

This method followed by the direct extraction procedure was performed exactly as described in Maniatis *et al.* (1982).

3) Low melting point gel

This method is based on the procedure described by Maniatis *et al.* (1982). The DNA was electrophoresed using low melting point agarose and visualised on a UV light box. The relevant band was cut from the gel using a scalpel and placed into a 1.5 ml Eppendorf tube. The tube was incubated in a 65°C water bath for 10-30 minutes to melt the agarose. The volume of agarose was measured and 2x volume of distilled water (preheated to 65°C) added. The mixture was then phenol extracted 3 times, phenol/chloroform extracted once and ethanol precipitated. The DNA was resuspended in an appropriate amount of distilled water.

I) DNA labelling

1) Nick translation of DNA

The nick translation method was based on the procedure used by Rigby *et al.* (1977). 1-2 µg of DNA was incubated in 0.1 volume of 10x nick translation buffer, 20 µCi α-^{32}P dCTP (3000 Ci/mmole), 25 ng/ml DNase I and 5 units of *E. coli* DNA polymerase I. The reaction was incubated at room temperature for 90 minutes. The labelled probe was separated from unincorporated nucleotides using a Sephadex G 50 column as described by Maniatis *et al.* (1982).

Cerenkov radiation was counted using a LKB Wallac liquid scintillation counter and the probe was denatured by boiling before use in hybridisations.

2) Random priming

The method used was based on the procedure described by Feinberg and Vogelstein (1984). The DNA fragment to be labelled was isolated from a low melting point agarose gel and the excised slice of gel was weighed and combined with 1.5 ml of distilled water per g of agarose. The mixture was heated to 95°C for 7 minutes then cooled to 37°C for 10-60 minutes and the concentration of DNA was determined on a minigel. 25 ng of DNA was combined with 0.2 volumes of 5x OLB, 500 µg/ml BSA, 25 µCi α-^{32}P dCTP and 2 units of Klenow fragment. The reaction was incubated at room temperature overnight. The labelled probe was isolated from unincorporated nucleotides and counted as described above.

2.9 Tissue culture

A) Culturing of cells as a monolayer

All cell lines were grown in plastic tissue culture flasks, 25 cm², 75 cm² and 150 cm² depending on the demand for the cell line. Every 3 days the medium was aspirated and fresh medium was added, 5 ml, 15 ml and 25 ml for the 3 flask sizes, repectively. The cells were subcultured when a confluent monolayer had formed. For subculturing of cells in 75 cm² flasks, medium was aspirated and the monolayer washed in 5 ml versene followed by 5 minutes incubation with 5 ml of a trypsin/versene mixture. After this incubation period 5 ml of medium was added and the cell suspension was transferred to a plastic universal and centrifuged at approximately 3 000

rpm for 5 minutes in an MSE minor bench centrifuge. The cell pellet was resuspended in 10 ml of medium and fresh tissue culture flasks containing fresh medium were seeded with 1 ml of cell suspension, hence cells were subcultured at 1:10 (except non transformed cell lines which were subcultured at 1:6). Cells were incubated at 37°C in a Wedco incubator with an humidified atmosphere of 5% carbon dioxide in air. Culturing of cells in 25 cm^2 and 150 cm^2 flasks was carried out in the same way using proportionate amounts of versene and trypsine/versene mixture.

B) Culturing of cells in suspension

The only cell line cultured in suspension was VR 19L. Fresh medium was added every 3 days and the cells were subcultured into fresh tissue culture flasks at a 1:6 dilution when necessary.

C) Storage of cells in liquid nitrogen

Stocks of the cell lines used during this project were stored frozen in liquid nitrogen until required. Cells were trypsinised as described in methods section 2.9 A and then resuspended to approximately 1 x 10^6/ml in medium containing 10% (v/v) FCS and 10% (v/v) dimethyl sulphoxide (filter sterilized with a flowpore filter unit). 1 ml aliquots of cell suspension were then placed into freezing ampoules, which were wrapped in several layers of tissue paper and aluminium foil (to ensure a slow decrease in temperature) and placed at -20°C for 2 hours, -70°C overnight and then stored in liquid nitrogen.

D) Recovery of cells stored in liquid nitrogen

Ampoules were removed from liquid nitrogen as required and the cells were thawed quickly at 37°C in a water bath. The cell suspensions were added to 10 ml of normal growth medium in a plastic universal, pelleted by centrifugation for 5 minutes at 3 000 rpm in an MSE minor bench centrifuge and resuspended in fresh medium prior to seeding to fresh tissue culture flasks as usual. After 24 hours the medium was replaced. The cells were maintained in continuous culture for at least 10 days before use.

E) Interferon-γ treatment of cells

The cells were grown until nearly confluent in normal growth medium at 37°C with an humidified atmosphere of 5% carbon dioxide in air. The medium was then aspirated and replaced with medium containing 100 U/ml of IFN-γ. The cells were then incubated for a further 72 hours at 37°C after which they were trypsinised and used as required.

F) Transfection of cells

This method is based on the procedure described by Graham and van der Eb (1973). 5 x 10^6 cells were plated into a 75 cm^2 tissue culture flask and incubated overnight at 37°C (5% carbon dioxide and humidified atmosphere) in 10 ml of normal growth medium. After this incubation period the DNA precipitate was prepared as follows. The DNA was dissolved in 0.5 ml of 2x HBS and a gentle stream of nitrogen was bubbled through as 0.5 ml of 0.25 M $CaCl_2$ was added dropwise. This mixture was left at room temperature for 30 minutes and was then added to the growth medium of the plated cells. The incubation at 37°C then continued for another 4-6 hours, then the medium was removed and 5 ml of 20% (v/v) glycerol in PBS (prewarmed to 37°C) was

added and incubated for 3-4 minutes at room temperature before it was removed and the cells were washed and fed with fresh medium. Culturing of cells was then continued as normal.

G) Treatment of cells with the antibiotic geneticin sulphate G418

Cells transfected with the gene for neomycin resistance were placed under selective pressure 4 days after the transfer of the DNA and this was maintained for at least 3 weeks. The cell lines used in this study were neomycin resistance selected by culturing in normal growth medium containing 0.31 mg/ml actual weight of geneticin sulphate G418 (A. G. Morris, pers. commun.).

H) Mitomycin C treatment

The cells were trypsinised as described in section 2.9.A then washed in PBS and pelleted by centrifugation as before. The cells were resuspended in 25 µg/ml mitomycin C in PBS and incubated at 37°C for 30-45 minutes in the dark. The cells were then washed 3 times in normal growth medium, counted using a Neubauer haemocytometer chamber and resuspended at the required cell concentration (Swain, 1980).

I) Cloning of cells

Cells were distributed into microtitre plates (96 wells) at 0.3 cells per well in 200 µl of normal growth medium. After 1 week the plates were examined and wells containing single colonies were selected. The medium was changed weekly in these wells and the colonies were subcultured by trypsinisation when large enough.

2.10 RNA manipulations

A) Extraction of mRNA from tissue culture cells

1) Isotonic lysis extraction

The method used was based on that described by Kumar and Lindberg (1972). To prevent RNA degradation all procedures except phenol extractions were carried out as quickly as possible at 4°C. The cells from a 150 cm^2 tissue flask were trypsinised as described in section 2.9.A, washed in 10 ml PBS, resuspended in 5 ml of isotonic lysis buffer and incubated on ice for 5 minutes. The cell suspension was then passed through a 21 gauge syringe needle to rupture the cell membrane and release the mRNA without damaging the nuclei. The cellular debris was then pelleted by centrifugation at 4 000 g for 5 minutes and the supernatant immediately mixed with equal parts of phenol extraction buffer, phenol and chloroform. The mixture was shaken and the phases separated by centrifugation at 4 000 g for 10 minutes at room temperature. The aqueous layer was phenol/chloroform extracted twice more and ethanol precipitated overnight at -20°C. After centrifugation the RNA pellet was washed in 70% (v/v) ethanol, dried in a vacuum dessicator, resuspended in distilled water and stored at -70°C. The RNA concentration was estimated by measuring the OD of the solution at 260 nm (1 OD_{260} unit was estimated to contain 40 µg/ml RNA).

2) Guanidinium isothiocyanate extraction

The method used for this procedure was exactly as described by Siggens (1987). The RNA was stored at -70°C.

B) Dot-blot hybridisation analysis of RNA

The application of RNA samples to nitrocellulose filters was carried out as described by Siggens (1987). The hybridisation of radiolabelled probes to the filters was performed by one of the following methods.

1) Hybridisation in the presence of formamide

This was carried out exactly as described by Siggens (1987).

2) Hybridisation in aqueous solution

The filter was soaked in 6x SSC for 5 minutes and then was incubated in aqueous hybridisation buffer (200 µl/cm² filter) at 65°C for 1 hour. At the end of this incubation period, the probe which had been previously denatured (boil 5 minutes, quench 5 minutes on ice) was added. The incubation then continued overnight at 65°C. The following washes were then carried out at 65°C. 3x SSC twice for 1 hour, 1x SSC twice for 1 hour. The filter was allowed to air dry, and after covering with cling film was exposed to a preflashed Fuji RX X-ray film at -70°C in the presence of a intensifying screen.

C) *In vitro* transcription

The method used is based on the procedure described by Melton *et al.* (1984).

1) Preparation of the template

Sequences to be transcribed were inserted in both orientations into the vector pGEM-1, which contains the promotors for both SP6 and T7 RNA polymerases separated by a multiple cloning site. The plasmid to be transcribed was linearised with a restriction enzyme with a unique site in

the polylinker down-stream of the coding sequence from the appropriate promotor. The DNA was then phenol/chloroform extracted twice, chloroform extracted once and ethanol precipitated. The resulting pellet was then resuspended in translation grade distilled water supplied with the Promega *in vitro* translation kit.

2) The transcription reaction

The reactions were typically 50 µl and contained 1-10 µg of template DNA, 0.2 volumes of the BRL 5x transcription buffer, 0.5 mM for each rNTP, 2 000 units/ml RNasin (ribonuclease inhibitor) and 300 units/ml SP6 or T7 polymerase. The reagents were added in that order and at room temperature to prevent precipitation of the DNA by the spermidine. The reaction was incubated at 40°C for 1 hour and then another aliquot of the enzyme was added and the incubation continued for another hour. At the end of this period DNase was added to a final concentration of 400 ng/ml and the reaction incubated at 37°C for 10 minutes to remove the DNA template. The RNA was then phenol/chloroform extracted and ethanol precipitated overnight at -20°C in the presence of 300 mM sodium acetate (instead of sodium chloride). The pellet which was washed in 80% (v/v) ethanol was resuspended in 50-200 µl of translation grade distilled water depending on the size of the pellet and was stored at -70°C.

2.11 Protein analysis

A) Indirect immunofluorescence staining of cell surface antigens and quantification by flow cytometry

1) Staining of cells

The cells to be stained were trypsinised as described in section 2.9.A, resuspended in medium, counted using a Neubauer haemocytometer chamber and the cell number was corrected to a concentration of 2.5×10^6. 100 µl of the cell suspension was aliquoted into the wells of a 96-well round-bottom microtitre plate. The cells covered with an adhesive plate sealer were pelleted at 1 000 rpm in an MSE mark II centrifuge and the supernatants carefully discarded. The cells were then resuspended in 100 µl of the first antibody at the saturating concentration (see below). This antibody was specific for the antigen of interest except in the case of the negative control where an irrelevant antibody was used to monitor the background levels of fluorescence by the cells. The microtitre plates were covered with a plate sealer and placed on a microtitre plate shaker for 30 minutes at 4°C. The cells were then pelleted as before, washed twice in 100 µl PBS and resuspended in 100 µl of the appropriate fluorescein-isothiocyanate (FITC) conjugated antibody specific for the species of origin of the first antibody used. The cells were then shaken for a further 45 minutes at 4°C, then washed twice more with 100 µl PBS, resuspended in 150 µl 0.5% (v/v) formaldehyde in PBS and stored at 4°C prior to analysis of fluorescence by flow cytometry. Cells were analysed within 24 hours. The flow cytometer analysis of cells was usually performed by myself, using the Becton-Dickinson FACStar flow cytometer. The flow cytometer was set up and the laser aligned with the sample stream using calibrite beads, as described in

the user's manual. Between 5 000 and 20 000 cells were analysed for each sample. All fluorescence data were collected in list mode using logarithmic amplification and analysed subsequently (see section 2 below). Forward scatter and side scatter signals were amplified by linear amplification.

Cells to be sorted on the flow cytometer were stained essentially as described above with the following exceptions. Staining was carried out in a plastic universal on 6 x 10^6 cells. 2 ml of the first and second antibody were used and 10 ml of PBS was used during the washes. The cells were not incubated in 0.5% (v/v) formaldehyde in PBS but were immediately sorted on the flow cytometer as described in the user's manual, under aseptic conditions, sterilizing the sorter with 70% ethanol.

2) Analysis of data

Data were analysed using the Becton-Dickinson Consort-30 computor program. Histograms of number of cells per channel against fluorescence intensity channel were plotted.

3) Titration of antibody preparations

In order to determine the highest dilution of antibody which, when used in the above protocol for indirect immunofluorescence staining, gave maximal staining of the antigen under investigation, the antibody was titrated as follows. The appropriate cell type was stained as described above with a dilution series of the antibody and a saturating concentration of the appropriate FITC-conjugated antibody (titrated previously). The cells were then analysed by flow-cytometry. To determine the highest dilution of antibody which gave maximal staining of the antigen (above which the fluorescence of cells became dependant upon the dilution of antibody) histograms were plotted of fluorescence intensity channel against number of cells per channel, and correlated with the dilution of antibody used.

B) Labelling of polypeptides

Cells to be labelled were seeded into 25 cm^2 flasks (2.5×10^6) and incubated in their normal growth medium at 37°C (5% carbon dioxide, humidified) overnight. The next day the medium was replaced with 1 ml of methionine-free medium and incubation was continued for a further 1-5 hours before labelling with ^{35}S-methionine (50 µCi/ml, 0.5 ml per flask) overnight. The cells were trypsinised as described in section 2.9.A then washed twice with 1 mM PMSF in PBS and the cell pellet was lysed in 100 µl of 1 mM PMSF in PBS and 10 µl of 20% (v/v) Nonidet P-40 in distilled water

on ice for 30 minutes. The cellular debris was pelleted by centrifugation at 10 000 g for 3 minutes and the supernatant was then either analysed immediately by SDS/PAGE (section 2.11.C) or the polypeptides were immunoprecipitated as follows.

C) Radioimmunoprecipitation

This method was based on the procedure described by Kessler (1980). Cell lysates were mixed with 50 µl of Immuno-precipitin (formalin fixed *Staphlococcus aureus*, which had been previously pelleted and resuspended in the same volume of RIP buffer) and 10 µl of foetal calf serum (heat treated to destroy anti-viral antibody) and incubated on ice for 30 minutes. The bacteria were pelleted by centrifugation at 10 000 g for 2 minutes and the supernatant incubated with 5 µl of the appropriate antibody at 4°C for 3 hours to overnight. The immune complexes were collated by incubation with 50 µl of Immuno-precipitin (in RIP buffer) on ice for 30 minutes followed by centrifugation at 10 000 g for 1 minute. The complexes were washed three times by resuspension in 1 ml of ice-cold RIP wash buffer followed by centrifugation for 30 seconds at 10 000 g. The pellets were finally resuspended in 40 µl of 5x SDS/PAGE sample buffer, then stored at -20°C or analysed immediately on a polyacrylamide gel. Prior to loading onto a gel the samples were boiled for 5 minutes and centrifuged at 10 000 g for 1 minute to remove the Immuno-precipitin.

D) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE)

The discontinuous buffer system of Laemmli (1970) was used. Single concentration resolving gels were used in the analysis of labelled and unlabelled polypeptides, typically 10% (w/v) acrylamide, with 6% (w/v) acrylamide stacking gels. The composition of the gel solutions are shown in section 2.6. The gels were formed using 250 x 200 mm plates with 1.5 mm spacers and electrophoresed in a Bio-Rad vertical slab gel apparatus as described by the manufacturer with SDS/PAGE running buffer at 30 V overnight or 70 V for 3 hours (at which point the dye front should reach the bottom the gel).

E) Preparation of samples for electrophoresis

Immunoprecipitated samples were prepared as described in section 2.11.C, *in vitro* translated proteins were prepared as described in 2.11.I, all others were prepared as follows. 0.2 volume of 5x SDS/PAGE sample buffer was added to each sample and they were heated to 90°C for 3 minutes in a boiling water bath and then quenched on ice before loading. All radiolabelled and unlabelled markers were also treated in this way before loading. The radiolabelled markers used were a commercial preparation of ^{14}C-labelled methylated proteins: lysozyme, 14.3 kD; β-lactoglobulin, 18.4 kD; α-chymotrypsinogen, 25.7 kD; ovalbumin, 46.0 kD; BSA, 68.0 kD; phosphorylase b, 97.4 kD; myosin (H-chain), 200.0 kD. The unlabelled markers used were a commercial preparation of the following proteins: lysozyme, 14.3 kD; trypsin inhibitor, 20.1 kD; carbonic dehydrogenase, 29.0 kD; alcohol

dehydrogenase, 38.9 kD; catalase, 58.1 kD; phosphorylase b, 97.4 kD and myosin (H-chain), 200.0 kD.

F) Fluorography and autoradiography

Fluorography, or impregnation of gels with a fluor, allows the detection of ^{3}H-labelled proteins when exposed to X-ray film, and increases the sensitivity of X-ray film to ^{35}S labelled proteins. Gels were immersed with gentle agitation in gel fixing solution for 60 minutes at room temperature and then were transferred to the fluorographic reagent Amplify for a further 30 minutes. The gels were then dried under vacuum for 1.5 hours using a Bio-Rad gel drier and exposed to a preflashed Fuji RX X-ray film at -70°C with an intensifier screen.

H) Western blotting

This method was based on that described by Towbin *et al.* (1979).

1) Transfer of proteins separated in a polyacrylamide gel to nitrocellulose

The gels were equilibrated with Western blot transfer buffer for 30 minutes and then placed on Whatman 3MM paper on a fibre pad resting on half of a gel holder of the Bio-Rad trans-blot apparatus. A sheet of nitrocellulose, cut to the same size as the gel, was placed on top, followed by 3MM paper and another fibre pad (all soaked in transfer buffer). The gel holder was inserted into the tank containing transfer buffer with the nitrocellulose towards the anode. Transfer was performed overnight at 30 V followed by 30 minutes at 70 V. The apparatus was dis-assembled and the nitrocellulose was stained for 5 minutes with Ponceau S stain. Destaining was carried out with

distilled water and the positions of the standard polypeptides marked with a pencil. Complete destaining was achieved by several washes in distilled water. Blocking of non-specific binding was carried out using 5% (w/v) defatted dried milk (Marvel) in PBS at 37°C for 1 hour or at 4°C overnight. The filter was washed at room temperature five times for 5 minutes in 0.1% (v/v) Tween 20 in PBS.

2) Detection of antigen-antibody complexes using a horseradish peroxidase colour reaction.

The filter was incubated at 4°C for 3 hours to overnight with the first antibody (at the appropriate dilution) in 5% (w/v) defatted dried milk in PBS then washed as above in 0.1% (v/v) Tween in PBS, before incubation in biotinylated second antibody (directed against the first antibody) diluted 1 to 500 in 1% (w/v) defatted dried milk in PBS for 1 hour at room temperature. The filter was washed again, then incubated in biotinylated peroxidase streptavidin complex diluted 1 to 300 in 1% (w/v) defatted dried milk for 15-30 minutes at room temperature followed by a further three 5 minute washes as described before and three rapid washes in PBS. Bound peroxidase activity was demonstrated by either a brown or grey precipitate on the addition of the diaminobenzidene developing solution or the Bio-Rad HRP developing solution, respectively. Once the desired band intensity was achieved, the reaction was stopped by washing in tap water repeatedly over a 30 minute period. The blot was dried overnight on Whatman 3MM paper, storing in darkness, before photographing.

I) *In vitro* translation of proteins

RNA was *in vitro* translated using the New England Nuclear reticulocyte lysate translation kit following the manufacturers instructions (for more information see Sambrook *et al*, 1989). At the end of the incubation period each sample was divided into two, one half was immunoprecipitated (2.11.C) and then both halves were analysed by SDS/PAGE (section 2.11.D).

2.12 Immunological procedures

A) Inoculation of mice

1) Intraperitoneal

The mice were placed under light anaesthesia, the abdominal walls were then washed with ethanol and 100 µl of the appropriate cell suspension were administered intraperitoneally using a 1 ml Sterilin syringe with a 26 gauge needle.

2) Subcutaneous

The mice were placed under light anaesthesia, the lower right legs were then washed with ethanol and 100 µl of the appropriate cell suspension were administered subcutaneously.

B) Cytotoxicity of MSV/MLV specific effector T lymphocytes

1) Preparation of MSV/MLV-specific effector T lymphocytes

Mice were immunised and boosted 7-14 days later by intraperitoneal injection of 3-5 x 10^6 mitomycin C and IFN-γ treated MSV/MLV infected cells as described in section 2.12.A. At day 7 post boost 3 mice were killed by cervical dislocation and the spleens aseptically removed. The spleens were placed into a 6 cm petri-dish containing MLTC medium and converted to a cell suspension by gently teasing with two scalpels. The cell suspension was transferred to a plastic universal and was converted to a single cell suspension by pipetting up and down in a 10 ml pipette. The cell suspension was then divided between two 150 cm^2 plastic flasks and 2-3 x 10^6 of mitomycin C and IFN-γ treated MSV/MLV infected cells were added to each flask. 50 ml of MLTC medium was added to each flask and they were incubated at 37°C in a Wadco incubator with an humidified atmosphere of 5% carbon

dioxide in air. The cells were harvested at day 5 post-culture for the cytotoxicity assay. The cells grow in suspension and so are harvested by centrifugation of the cell suspension in a plastic universal at 3 000 rpm for 5 minutes in a MSE minor bench centrifuge. The cells are resuspended in fresh MLTC medium and centrifuged on a layer of lymphoprep following the manufacturer's instructions, to remove non-viable cells, debris, and red blood cells. The cells were then counted using a Neubauer counting chamber and resuspended in fresh MLTC medium at the appropriate cell concentrations for the assay.

2) Radiolabelling of target cells

The target cells were trypsinised, resuspended in fresh medium, counted using a Neubauer haemocytometer chamber and tissue culture petri dishes (6 cm diameter) were seeded with 3×10^6 cells. The medium was adjusted to 3 ml and 50 µCi ^{51}Cr (sodium chromate) was added to each petri dish and the cells incubated overnight at 37°C (5% carbon dioxide, humidified).

3) The assay

16 hours post-labelling the target cells were trypsinised, then washed and resuspended in fresh medium, before counting on a Neubauer haemocytometer chamber. The cell concentrations were adjusted to 1×10^5/ml and 100 µl aliquots added to the wells of 96 well round bottomed microtitre plates in 5x replicae. 100 µl aliquots of effector T lymphocytes were then added to the wells to give target:effector ratios within the range of 1:1 to 1:50 (typically, 1:3, 1:10 and 1:30). Additionally, 100 µl aliquots of 1 M hydrochloric acid or medium were added to control wells of each target cell type in order to assess total release and spontaneous release of ^{51}Cr from the cells, respectively. The plates were incubated at 37°C (5% carbon

dioxide, humidified) for 4-6 hours and then were covered and centrifuged at 1 000 rpm for 1 minute to pellet the cells and facilitate dissolution of ^{51}Cr from lysed cells. 100 µl of each supernatant was removed and placed in a 4 ml plastic test-tube which was sealed with paraffin wax. ^{51}Cr-released was determined using an LKB gamma counter and specific release was calculated as described in the following section.

4) Analysis of data

Specific release was calculated using the formula:

$$\% \text{ lysis} = \frac{(\text{release in presence of effector} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100$$

In each experiment the cytotoxicity of the effector T lymphocytes against MSV/MLV infected cells (KC3H or MoSVC3H) and uninfected fibroblasts (C3H10T½) or transformed fibroblasts (C3H201) was assessed in order to monitor the specificity. Cytotoxicity against YAC-1 cells, a cell line highly susceptible to natural killer cell lysis (Kiessling *et al.*, 1975; Chervenak and Wolcott, 1988), was assessed in assays using Ki-MSV/MLV specific effector T lymphocytes, to monitor levels of natural killer cell activity in the effector population.

C) Tumour growth studies

The immunization protocol and the cell concentration of the tumour cell inoculum varied depending on the experiment therefore these details are recorded with the results of each experiment. 10 days post tumour cell inoculation and every two days thereafter until sacrificed, the mice were examined and the number of tumours scored by palpation.

Chapter 3 The use of the HLV *gag* and *env* genes to generate tumour cell lines expressing specific tumour antigens

3.1 Introduction

In this study, *gag* and *env* expression vectors were constructed using standard molecular biology techniques (section 3.2). After construction the vectors were transfected into the *ras* transformed fibroblast cell line C3H201 and the transfected cell populations were examined for *gag* and *env* expression by indirect immunofluorescence and flow cytometry analysis (section 3.3).

3.2 Construction of retroviral vectors expressing a specific HLV antigen

Recombinant retroviral vectors with exogenous genes replacing a portion of the viral genome have been successfully used by many groups to introduce genes into mammalian cells. Here, the vectors are designed in a similar way, using LTRs flanking the gene to provide the necessary transcription elements. The LTR 5' to the gene provides the tRNA binding site, promotor and enhancer sequences, and the cap site, whereas the 3' LTR supplies the necessary information for polyadenylation of the mRNA (see Figure 1.8). In this study, however, the vectors are used to express specific retroviral genes. The main reason for the now wide-spread use of retroviral vectors is the availability of retrovirus packaging cell lines which allow the production of replication-defective retrovirus vectors in the absence of helper virus (Joshi *et al.*, 1990). As discussed in the introduction, the vectors used in this study carry the packaging signal ψ (Mann *et al.*, 1983) thus enabling the RNA produced from these vectors to be packaged into virion when transcribed in a packaging cell line, such as ψ-2.

The Ki and Mo MLV clones used in the construction of the *gag* and *env* expression vectors do not contain complete LTRs. It was therefore necessary to construct complete LTRs for use in the expression vectors. The construction of a complete Ki LTR is shown in Figure 3.2a. and the methods used are described in section 2.8. The pUC Ki LTR AB plasmid DNA was digested with a range of restriction enzymes and the results from this analysis are shown in Figure 3.2b. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. Oligonucleotide A ligated to the 3' end of the LTR fragment reconstitutes the packaging site and provides a BamH I restriction enzyme site. Oligonucleotide B supplies sequence missing from 5' end including the inverted repeat and also provides a Sac I restriction enzyme site, thus enabling the complete LTR to be excised from pUC13 by double digestion with BamH I and Sac I. The complete Mo LTR used during the construction of the Mo expression vectors was prepared and supplied by G. Ward (University of Warwick).

Legend to figure 3.2a. Construction of the complete Ki long terminal repeat pUC Ki LTR AB. The cp2 Ki MLV clone (~2800 bp) was released from pAT153 by a Pst I digestion, then was further digested with spe I and the resulting Pst I/spe I LTR fragment (~650 bp) was isolated. pUC13 was digested with Pst I and BamHI, dephosphorylated with CIAP and the Pst I/spe I LTR fragment and the oligonucleotide A (Appendix A) were cloned into this vector via a three way ligtion to form pUC Ki LTR A. pUC Ki LTR A was then digested with Pst I and BamHI and the LTR fragment was isolated. Finally, pUC13 was digested with Sac I and BamHI, dephosphorylated with CIAP and the Pst I/BamHI LTR fragment oligonucleotide B (Appendix A) were cloned into this vector via a three way ligation to form the vector pUC Ki LTR AB, containing a complete Ki LTR.

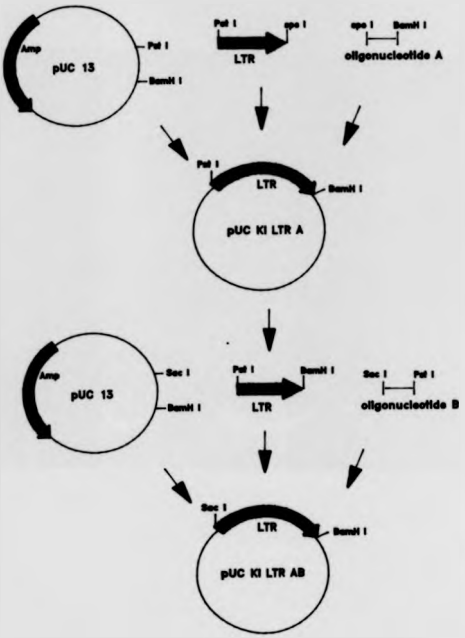


Figure 3.2a. Construction of the complete KI long terminal repeat pUC KI LTR AB

Legend to figure 3.2b. Restriction map analysis of the vector pNC K1 LTR AB. Plasmid DNA was restricted with Sac I/BamH I (a), Kpn I/Hha I (b), Kpn I/ Sac I (c), Kpn I/BamH I (d), Kpn I/EcoR I (e) and EcoRI (f). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with Sac I/BamH I would result in fragments of 0.70 and 2.68 kbp. See Addendix D

A) *gag* expression vectors

i) The Kirsten *gag* expression vector pUC Ki gag

The construction of pUC Ki gag is shown in Figure 3.2A.ia and the methods used are described in section 2.8. The pUC Ki gag plasmid DNA was digested with a range of restriction enzymes and the results from this restriction analysis are shown in Figure 3.2A.ib. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. It can be seen that a small amount of *pol* sequence (~350 bp) is present 3' to the *gag* gene in this vector. The published data, on the construction of similar vectors, has shown that the presence of such sequence does not interfere with the expression of the gene of interest in these vectors (Flyer *et al.*, 1983; Abastado *et al.*, 1985; Holt *et al.*, 1986). In order to identify proteins recognised by anti-Friend virus Tc, Holt *et al.* (1986) produced constructs that expressed the proteins encoded by either the Friend MLV *gag* or *sav* genes. The *gag* expression vector was constructed by the deletion of a Sac II fragment from a genomic clone. The resulting vector contains ~3 000 bp of the *pol* gene and ~600 bp of the *sav* gene located between the *gag* gene and the 3' LTR which supplies the information for polyadenylation. This vector is known to express the Friend *gag* polypeptides, $Pr65^{gag}$, $gPr80^{gag}$ and $gPr95^{gag}$ by radioimmunoprecipitation followed by SDS/PAGE, showing that the presence of the *pol* and *sav* sequence does not interfere with the transcription and translation of the *gag* gene of this vector. Therefore it is proposed that the *pol* sequence present in pUC Ki gag will not interfere with the expression of the *gag* gene. This claim is supported by evidence presented later in this thesis. Finally, it should be noted that the precursor protein $Pr65^{gag}$ is not cleaved into the core proteins P15, P12, P30 and P10 and this is discussed in detail in the next section.

Legend to figure 3.2A.ia. Construction of the Ki *gag* expression vector pUC Ki *gag* . The cp2 Ki MLV clone (~2800 bp) was released from pAT153 by a Pst I digestion then was further digested with Kpn I and Sac I. The resulting Kpn I/Sac I *gag* fragment (~2200 bp) was isolated. The vector pUC Ki LTR AB was digested with Sac I and BamHI, and the complete LTR (~700 bp) was isolated. Finally, pUC Ki LTR AB was digested with Kpn I and BamHI, dephosphorylated with CIAP and the cp2 Kpn I/Sac I fragment and Ki LTR AB Sac I/BamHI were cloned into this vector via three way ligation.

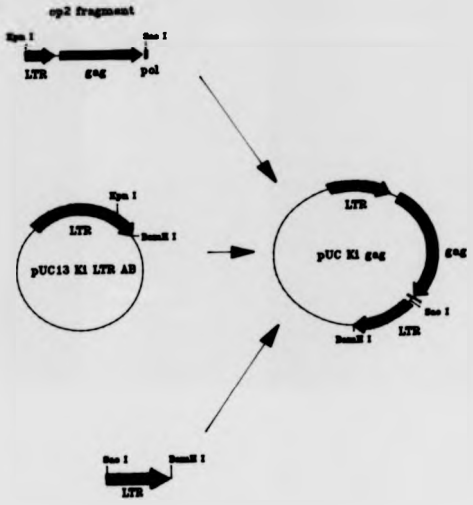
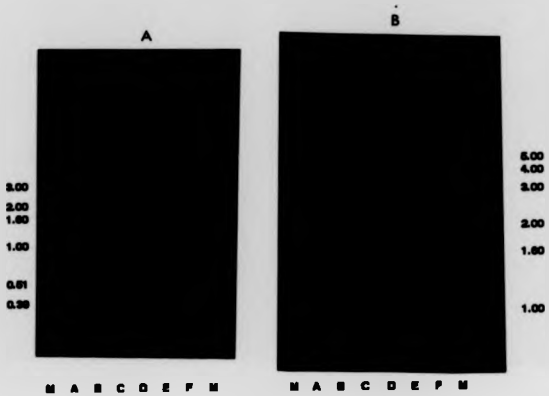


Figure 3.2A.ia. Construction of the K1 gag expression vector pUC K1 gag.



Legend to figure 3.2A.1b. Restriction map analysis of the expression vector pSC Ki gag2. Plasmid DNA was restricted with EcoR I/Hind III (a), Pst I (b), Pvu II (c), Kpn I (d), BamH I (e) and EcoR I (f). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with Pst I would result in fragments of 0.70, 2.80 and 2.68 kbp. See Appendix D.

ii) The Moloney *gag* expression vector pUC Mo gag

The construction pUC Mo gag is shown in Figure 3.2A.iia and the methods used are described in section 2.8. The pUC Mo gag plasmid DNA was digested with a range of restriction enzymes and the results from this analysis are shown in Figure 3.2A.iib. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. It can be seen that pUC Mo gag contains ~700 bp of *pol* sequence between the *gag* gene and the 3' LTR. Again this is not thought to interfere with the expression of the *gag* gene. Flyer *et al.* (1985) report the production of a Mo *gag* expression vector which was constructed in a similar manner to pUC Mo gag. When transfected into a mouse fibroblast cell line, this vector is reported to express the *gag* precursor protein $Pr65^{gag}$ (no $gPr80^{gag}$ and $gPr95^{gag}$ detected) thus supporting the use of this type of expression vector. Both Flyer *et al.* (1985) and Holt *et al.* (1986) observed no cleavage of the precursor protein $Pr65^{gag}$.

In MLV infected cells, the *pol* gene is expressed in the form of a large fusion protein, $Pr200^{gag\text{-}pol}$, by the translational read-through of a terminator codon at the end of the *gag* gene. It is now known that the *pol* portion of the *gag-pol* protein is processed in the assembling virion to form three mature products: a proteinase ($P14^{pol}$); the reverse transcriptase enzyme ($P80^{pol}$); and integrase ($P46^{pol}$), a protein responsible for the integration of the viral DNA (Goff and Lobel, 1987; section 1.6). The biochemical properties of the proteinase isolated from MLV virions have been known for some time, however the gene encoding the function was only recently identified. Crawford and Goff (1985) generated a Mo MLV mutant in the P14 domain by making a 126 bp deletion in the 5' portion of *pol*. This deletion preserved the reading frame down stream thus permitting expression

Legend to figure 3.2A.iia. Construction of the Mo *gag* expression vector pUC Mo *gag*. pZAP was digested with xba I and Hind III and the resulting fragment containing the *gag* gene (~5000 bp) was isolated. pUC13 Mo LTR (Appendix B) was also digested with xba I and Hind III, dephosphorylated with CIAP and the vector fragment was isolated. The two fragments were ligated to form pUC13 Mo LTR *gag*. This vector was then digested with stu I and Hind III (to remove the majority of the *pol* sequence), dephosphorylated with CIAP and the vector fragment was isolated (~5800 bp). A complete LTR was isolated from pUC13 Mo LTR by digesting with EcoRI, which was subsequently blunted with klenow, and then with Hind III. Finally, the LTR was cloned into the stu I/Hind III site of pUC13 Mo LTR *gag* via a two way ligation to form pUC Mo *gag*.

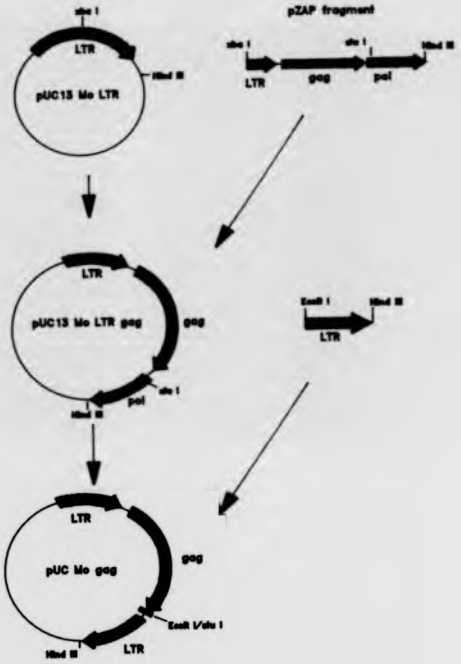
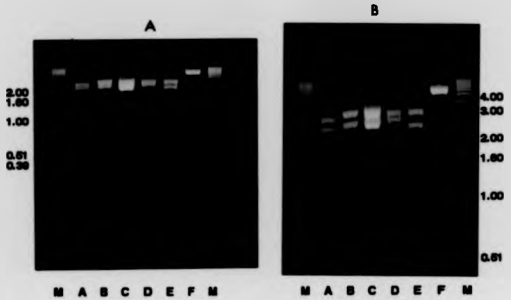


Figure 3.2A.IIa. Construction of the Mo gag expression vector pUC Mo gag.



Legend to figure 3.2A.11b. Restriction map analysis of the expression vector pHC Hu gag. Plasmid DNA was restricted with H/A (a), Kpn I (b), Pst I (c), Xba I (d), EcoR I/Hind III (e) and Hind III (f). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with Pst I would result in fragments of 0.18, 2.58 and 3.73 kbp. See Appendix O.

of the remaining portions of the *pol* gene. When NIH/3T3 cells were transfected with this mutant virus, they were induced to synthesize normal levels of *gag*, *gag-pol* and *env* precursors. However, no cleaving of the *gag* and *gag-pol* proteins occured in these cells, suggesting that the 5' part of the *pol* gene encoded the proteinase function and that this enzyme normally was responsible for cleaving both of these precursors. The vectors constructed by Flyar *et al.* (1985) and Holt *et al.* (1986) similarly lack a functional *pol* gene, and when expressed in a mouse cell line, no processing of $Pr65^{gag}$ has been observed. pUC Ki gag and pUC Mo gag are likewise missing a functional *pol* gene, therefore no cleavage of the precursor protein $Pr65^{gag}$ is expected.

B) *env* expression vectors

i) The Kirsten *env* expression vector pUC Ki env

The construction of pUC Ki env is shown in Figure 3.2B.ia and the methods used are described in section 2.8. The pUC Ki env plasmid DNA was digested with a range of restriction enzymes and the results from this analysis are shown in Figure 3.2B.ib. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. This *env* expression vector uses the transcriptional elements of the retroviral LTR in a similar manner as described for pUC Ki gag and pUC Mo gag. In addition, the splice donor and acceptor sites which are utilised to produce the spliced mRNA, from which the *env* products are translated in MLV infected cells, are also deployed in this construct to allow expression of the *env* products. This useful feature has been used by many groups when devising retroviral expression vectors. In most cases vectors are designed to carry two genes; one gene being translated from the primary transcript and the other from the spliced mRNA. Frequently, one of the genes provides a selectable

Legend to figure 3.2B.1a. Construction of the Ki *env* expression vector pUC Ki *env*. The cpl Ki MLV clone (~5100bp) was released from pAT153 by a Pst I digestion, then was further digested with Pvu II and the resulting Pvu II/Pst I fragment containing the complete *env* gene and the splice acceptor site (~2300 bp) was isolated. pUC Ki LTR AB was digested with BamHI which was blunted with klenow and then subsequently digested with xba I and dephosphorylated with CIAP. Finally, pUC Ki LTR AB was digested with Pst I and xba I and the LTR fragment was isolated and cloned into the dephosphorylated vector with the cpl Pvu II/Pst I fragment via a three way ligation.

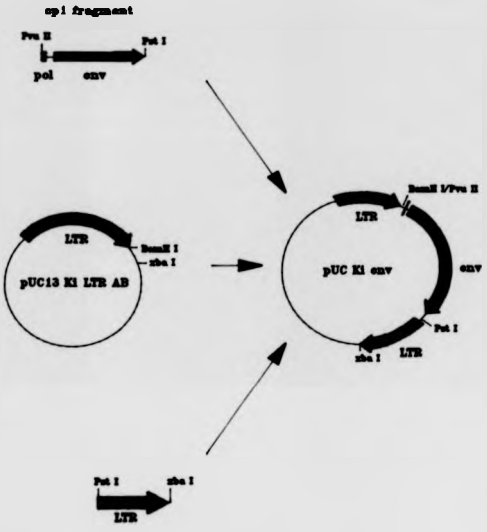
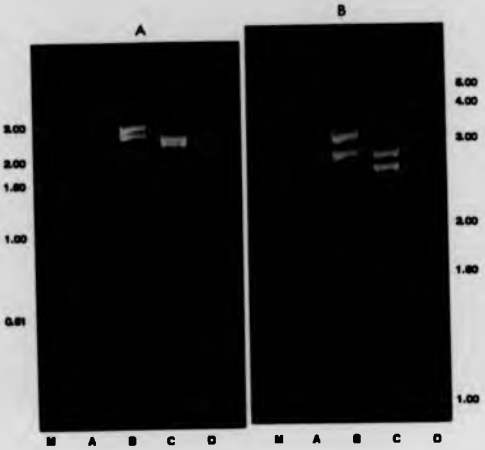


Figure 3.2B.ia. Construction of the K1 env expression vector pUC K1 env



Legend to figure 3.2B.1b. Restriction map analysis of the expression vector pBC K1 env. Plasmid DNA was restricted with Hind III (a), Kpn I (b), Pst I (c), Sac I (d). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with Pst I would result in fragments of 0.70, 3.00 and 2.68 kbp See Appendix D.

phenotype such as resistance to the antibiotic G418 and the other is a nonselectable gene whose transfer is desired (Capka *et al.*, 1984; Bowtell *et al.*, 1988). In pUC Ki *sav* only one gene is present and the expression of this gene is achieved via the spliced mRNA. The splice donor site AGGTAAG (Horton *et al.*, 1984) is present in the LTR 5' to the *sav* gene. The splice acceptor site, CUCUCCAAG for the closely related AKR murine leukemia virus (Herr, 1984), is in a highly conserved sequence preceding the 3' end of *pol* and is ~300 bp down stream from the Pvu II site used during the construction of this vector.

Flyer *et al.* (1983) report the construction of two Mo *sav* expression vectors similar to pUC Ki sav. Although they both utilised the splice donor and acceptor sites, the vectors had different amounts of intervening sequence between the 5' LTR and the *sav* gene. It was found that the vector with the least intervening sequence expressed the *sav* gene product (pr80) more efficiently when transfected into mouse BALB/c-3T3 cells. This was taken into account when designing the expression vector pUC Ki sav, such that the restriction enzyme site resulting in the least sequence between the 5' LTR and the start of the *sav* gene was used. Additionally, Flyer *et al.* (1983) report that in the absence of the *pol* gene the *sav* precursor protein was still cleaved into the two products gp70 and p15E, the significance of which is discussed below.

ii) The Moloney *env* expression vector pUC Mo env

The construction of pUC Mo env is shown in Figure 3.2B.iia and the methods used are described in section 2.8. The pUC Mo env plasmid DNA was digested with a range of restriction enzymes and the results from this analysis are shown in Figure 3.2B.iib. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. This vector was constructed in a similar manner to pUC Ki env, with the splice donor site AGGTAAG and splice acceptor site CTGCTGCAG (Shimnick, 1981) being used to express the *env* gene products. Only a small amount of *pol* sequence (~370 bp) is present between the 5' LTR and the *env* gene; and the splice acceptor site is contained within this sequence. As previously discussed, Flyer *et al.* (1983) designed Mo *env* expression vectors similar to pUC Ki env and pUC Mo env. These vectors, which also lack the majority of the *pol* gene, produced the *env* precursor protein when transfected into NIH/3T3 cells which was cleaved into the two products gp70 and p15E. This cleavage in the absence of a complete *pol* gene is also observed by Crawford and Goff (1985) in their studies with Mo MLV *pol* deletion mutants. The latter found that in the absence of the proteinase product of the *pol gene*, the *env* precursor was cleaved into the products gp70 and P15E, whereas the *gag* and *gag-pol* precursors were not (see above). They thus propose that the processing of the *env* precursor is probably mediated by host enzymes and not the *pol* proteinase. It is also reported that the P15E protein was not processed to the smaller P12E, indicating that this cleavage was dependent on the *pol* proteinase.

Legend to figure 3.2B.iia. Construction of the Mo *env* expression vector pUC Mi *env*. pZAP was digested with Sac I and the resulting fragment containing the complete *env* gene and splice acceptor site (~5700 bp) was isolated. pUC13 Mo LTR (Appendix B) was digested with Sac I and dephosphorylated with CIAP. The pZAP fragment was cloned into the Sac I site of pUC13 Mo LTR via a two way ligation to form pUC13 Mo LTR env (the orientation was checked with a Hind III digest). This vector was subsequently digested with Bgl II (to remove the majority of *pol* sequence), blunted with klenow, further digested with EcoRI and then dephosphorylated with CIAP. A complete LTR was isolated from pUC13 Mo LTR following an EcoRI and Hinc II digestion and was cloned into the Bgl II (blunt)/EcoRI site of pUC13 Mo LTR env via a two way ligation to form pUC Mo *env*.

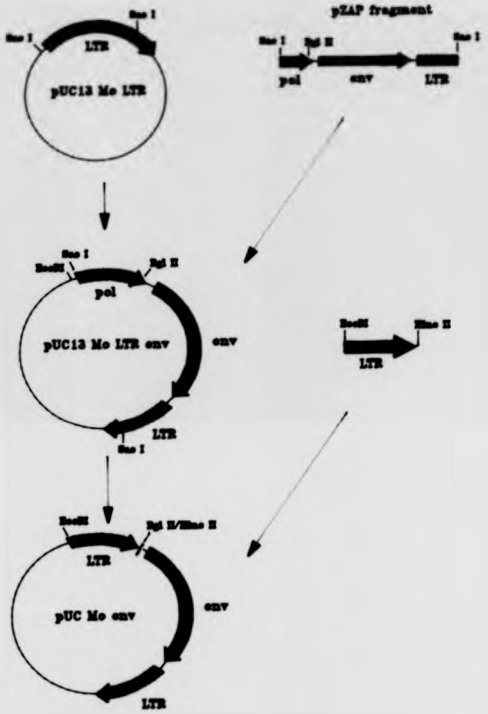
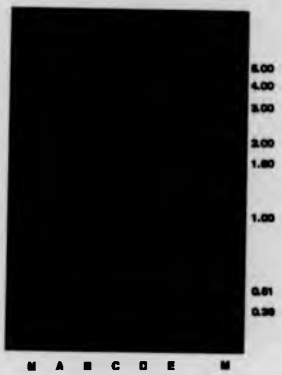


Figure 3.2B.iia. Construction of the Mo env expression vector pUC Mo env



Legend to figure 3.2B.iib. Restriction map analysis of the expression vector pBC Ho env. Plasmid DNA was restricted with BamH I(a), Sac I (b), Hind II/EcoR I (c), Kpn I (d), N/A (e). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with BamH I would result in fragments of 1.90, 1.20 and 3.40 kbp. See Appendix D

3.3 Flow cytometry analysis of cells transfected with the *gag* and *env* expression vectors

The expression vectors discussed above were transfected into the *ras* transformed embryo fibroblast cell line C3H201 as described in section 2.9F. The DNA used for each transfection was of large scale plasmid preparation quality and had been isolated on a caesium chloride gradient. In each case the plasmid DNA was linearised by cutting within the pUC13 sequence prior to use in a transfection. Control transfections were carried out to ensure that the transfection procedure was working efficiently. This was achieved by transfecting the embryo fibroblast cell line C3H10T½ with plasmid DNA containing the Ki *ras* oncogene flanked by two functional LTRs. This plasmid was supplied by G. Ward and was constructed using the KCC7 clone supplied by J. D. Norton (section 2.1). Cells that have been successfully transfected are easily identified as they become morphologically transformed when the *ras* gene is expressed.

After transfection with the expression vectors the cells were cultured for a further 5-6 days and then were examined for *gag* or *env* expression by indirect immunofluorescence followed by flow cytometry analysis (section 2.11A). Data were analysed using the Becton-Dickinson Consort-30 computer program and histograms of number of cells per channel against fluorescence intensity channel were plotted. In each case, the appropriate positive and negative control cells were stained together with the transfected cell line. Staining with the FITC-conjugated second antibody alone was also carried out. All transfected cell lines are tabulated in Appendix B.

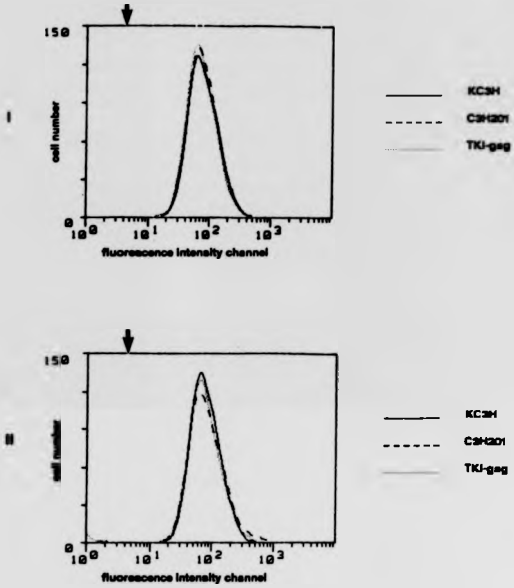
A) *gag* expression vectors

i) The Kirsten *gag* expression vector

When transfected with pUC Ki gag, the cell line is referred to as TKi-gag. The appropriate positive and negative control cell lines used for the indirect immunofluorescence staining of TKi-gag were KC3H and C3H201, respectively (section 2.3). The C3H201 cell line, which is derived from the embryo fibroblast cell line C3H10T½ by infection with helper-free Ki-MSV, is transformed by the Ki-*ras* oncogene but expresses no MLV proteins. The KC3H cell line, derived from the C3H10T½ cell line by infection with the Ki-MSV/MLV complex, is likewise transformed by the Ki-*ras* oncogene but also expresses the Ki MLV polypeptides.

To examine the expression of Ki *gag* polypeptides by TKi-gag, the transfected cell line and the control cells were stained with the anti-Ki-gag antibody (2.5C) by indirect immunofluorescence and then were analysed by flow cytometry. The anti-Ki-gag antibody was used at a range of dilutions (0 to 1/10 000) and the FITC-conjugated second antibody (rabbit-anti-goat) was used at a saturating concentration (titrated previously). The results of the flow cytometry analysis of cells stained with anti-Ki-*gag* at a dilution of 1/100 are shown in histogram I of Figure 3.3A.i. At this dilution (and all others) there was no significant difference in staining between TKi-gag, KC3H and C3H201. At the lower dilutions there appears to be strong background staining with this antibody for all three cell lines (as shown for the dilution 1/100). The non-specific background staining decreases with antibody concentration, however the decrease is the same for each cell line. In an attempt to reduce this non-specific staining the antibody was preabsorbed with the negative control cell line C3H201 (1×10^7 cells/ml of antibody). This preabsorption treatment had no effect on the high background staining (shown in histogram II of Figure 3.3A.i).

Figure 3.3A.I Flow cytometry analysis of cells expressing KI gag polypeptides



The arrows indicate the position of the C3H201 cells stained with FITC rabbit-anti-goat alone.

Cells were stained with the anti-KI-gag antibody by indirect immunofluorescence as described in the method section 2.11.A. 5000 cells were analysed in each case.
I. Anti-KI-gag antibody was used at a dilution of 1/100.
II. Anti-KI-gag antibody was preabsorbed with C3H201 cells and used at a dilution of 1/100.

Mouse liver powder was also used to preabsorb this antibody but was similarly unsuccessful (data not shown).

ii) The Moloney *gag* expression vector

When transfected with pUC Mo gag, the cell line is referred to as TMo-gag. The appropriate positive and negative control cell lines used for the indirect immunofluorescence staining of TMo-gag were MoSVC3H and C3H201, respectively (section 2.3). The C3H201 cell line, as discussed above, expresses no MLV proteins. The MoSVC3H cell line, derived from the C3H107% cell line by infection with the Mo-MSV/MLV complex, is transformed by the Mo-*mos* oncogene and also expresses the Mo MLV polypeptides.

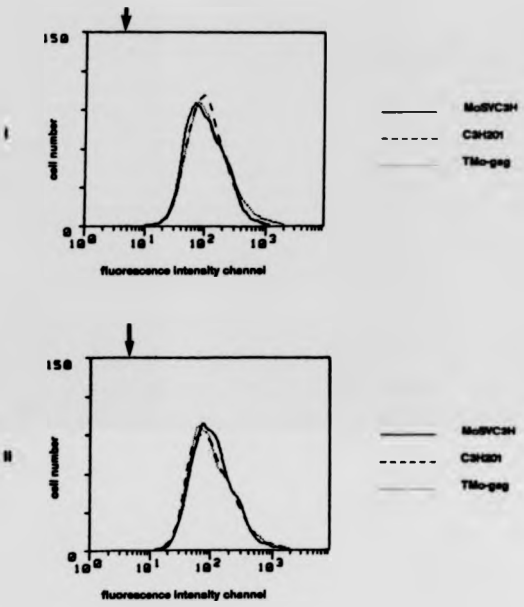
To examine the expression of Mo *gag* polypeptides by TMo-gag, the transfected cell line and the control cells were stained with anti-Mo-gag antibody (2.5C) by indirect immunofluorescence. This antibody was used at a range of dilutions (0 to 1/5 000) and the FITC-conjugated second antibody (rabbit-anti-goat) was used at a saturating concentration (titrated previously). The results of the flow cytometry analysis of cells stained with anti-Mo-*gag* at a dilution of 1/100 are shown in histogram I of Figure 3.3A.ii. It can be seen from this figure that strong non-specific staining is observed in the same way as described above. Again preabsorption of this antibody had no effect on the high non-specific staining (histogram II, Figure 3.3A.ii).

B) *env* expression vectors

i) The Kirsten *env* expression vector

When transfected with pUC Ki env, the cell line is referred to as TKi-env. The appropriate positive and negative control cell lines used for the indirect immunofluorescence staining of TKi-env were KC3H and C3H201, as

Figure 2.3A.II. Flow cytometry analysis of cells expressing Mo-gag polypeptides



The arrows indicate the position of the C3H3D1 cells stained with FITC rabbit-anti-goat sera.

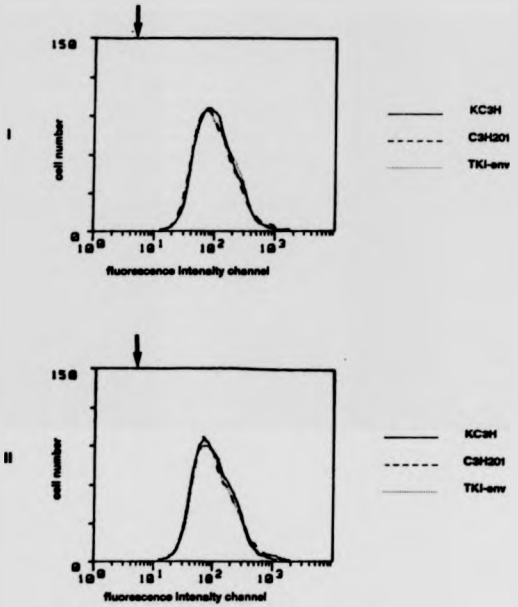
Cells were stained with the anti-Mo-gag antibody by indirect immunofluorescence as described in the method section 2.11.A. 8000 cells were analysed in each case.
I. Anti-Mo-gag antibody was used at a dilution of 1/100.
II. Anti-Mo-gag antibody was preabsorbed with C3H3D1 cells and used at a dilution of 1/100.

for section 3.3A.i. To examine the expression of Ki *env* polypeptides by TKi-env, the transfected cell line and the control cells were stained with anti-Ki-env antibody (2.5C). This antibody was used at a range of dilutions (0 to 10 000) and the FITC-conjugated second antibody (rabbit-anti-goat) was used at a saturating concentration (titrated previously). The results of the flow cytometry analysis of cells stained with anti-Ki-*env* at a dilution of 1/100 are shown in histogram I of Figure 3.3B.i. Again, strong non-specific staining is observed and preabsorption of this antibody with the negative control cell line or mouse liver powder has no significant effect (for example, see histogram II of Figure 3.3B.i).

ii) The Moloney *env* expression vector

When transfected with pUC Mo env, the cell line is referred to as TMo-env. The appropriate positive and negative control cell lines used for the indirect immunofluorescence staining of TMo-env were MoSVC3H and C3H201, as for section 3.3A.ii. To examine the expression of Mo *env* polypeptides by the transfected cell line, TMo-env and the control cells were stained with anti-Mo-env antibody (2.5C). This antibody was used at a range of dilutions (0 to 5 000) and the FITC-conjugated second antibody (rabbit-anti-goat) was used at a saturating concentration (titrated previously). The results of the flow cytometry analysis of cells stained with anti-Mo-*env* at a dilution of 1/100 are shown in histogram I of Figure 3.3B.ii. A high level of background staining occurs with this antibody, again with no difference in the level of staining observed between the three cell lines. Attempts to improve this staining by preabsorption (as described for the previous three antibodies) were unsuccessful, as is illustrated in histogram II of Figure 3.3B.ii.

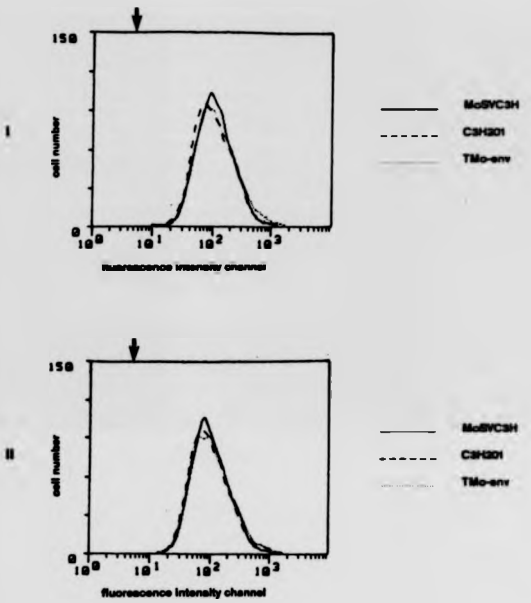
Figure 2.38.I. Flow cytometry analysis of cells expressing KI env polypeptides



The arrows indicate the position of the C3H201 cells stained with FITC rabbit-anti-goat alone.

Cells were stained with the anti-KI-env antibody by indirect immunofluorescence as described in the method section 2.11.A. 6000 cells were analysed in each case.
I. Anti-KI-env antibody was used at a dilution of 1/100.
II. Anti-KI-env antibody was preabsorbed with C3H201 cells and used at a dilution of 1/100.

Figure 3.3B.II. Flow cytometry analysis of cells expressing Mo-env polypeptides



The arrows indicate the position of the C3H201 cells stained with FITC rabbit-anti-goat alone.

Cells were stained with the anti-Mo-env antibody by indirect immunofluorescence as described in the method section 3.11.A. 8000 cells were analysed in each case.
I. Anti-Mo-env antibody was used at a dilution of 1/100.
II. Anti-Mo-env antibody was preabsorbed with C3H201 cells and used at a dilution of 1/100.

3.4 Discussion

It was originally hoped that cells successfully transfected with the *gag* and *env* expression vectors could be selected by sorting on a flow cytometer, after staining by indirect immunofluorescence. All cells showing greater fluorescence than the appropriate negative control cell line would be collected; as the higher fluorescence would be attributed to the expression of the antigen of interest. Initially, these observations appear to contradict those of Flyer *et al* (1983, 1985). They report the use of these antibodies to quantify, by indirect immunofluorescence and flow cytometry analysis, the level of *gag* and *env* expression in their transfected cell lines. However, it should be noted that their cells are of a different origin than those used in this study and this may explain the discrepancy. It can be seen from the results above that the antibodies used to detect either *gag* or *env* expression are not suitable for this use, thus alterative selection strategies have been designed (see chapters 4 and 6).

Chapter 4 The use of neomycin resistance vectors to select successfully transfected cells

4.1 Introduction

It was shown in chapter 3 that cells successfully transfected with the *env* and *gag* expression vectors cannot be selected by sorting on a flow cytometer after staining by indirect immunofluorescence. In this chapter the use of neomycin resistance as an alternative selection strategy is described.

The bacterial transposon Tn5 encodes a gene (*neo*^{r}) whose protein product, an aminoglycoside 3'-phosphotransferase (APH) II, confers resistance to the kanamycin-neomycin group of antibiotics (Southern and Berg, 1982). Neomycin and kanamycin are bacterial antibiotics which interfere with prokaryotic ribosomes. Mammalian cells are not affected by these antibiotics, however an analogue of these drugs does effect eukaryotic ribosomes. It is this analogue G418, which is purchased from Gibco as Geneticin, that is used in this study (section 2.9G), yet by convention the selection system in eukaryotes is still referred to as neomycin resistance. The concentration of Geneticin required to kill untransfected cells was previously determined by A. G. Morris (personal communication). After the addition of Geneticin, untransfected cells generally died within one week allowing colonies of resistant cells to form by 10-14 days. The resistant cells were cultured in Geneticin for at least 3 weeks following selection thus ensuring that all cells present in the culture were expressing the neomycin resistance gene. The cell lines were also periodically checked for resistance to ensure that the *neo*^{r} gene had not been lost during culturing, as observed occasionally by other groups (discussed in some detail later).

Early studies employing the *neo*^{r} gene as a selection marker for vectors in prokaryotes (Herrmann *et al.*, 1978) and eukaryotes (Jimenez and Davies, 1980; Southern and Berg, 1982) used unnecessarily large fragments of DNA as only the approximate position of the *neo*^{r} gene in Tn5 was known. Following nucleotide sequencing, the exact location of the gene is now known (Beck *et al*, 1982) and this has been used for the construction of more defined vectors at the nucleotide level (for example, Boulter and Wagner, 1987). Due to the suitability of restriction enzyme sites, the vectors utilised in this work were pSV2neo (Southern and Berg, 1982) and pNEO (see Pharmacia catalogue). pSV2neo was produced by inserting the *neo*^{r} gene into the mammalian transcription unit of the pSV plasmid vector (Southern and Berg, 1982). In this arrangment the SV40 early promotor is 5', and an intron and polyadenylation signal are 3', to the *neo*^{r} gene. This vector, which is expressed when transfected into mammalian cells without further alteration, is used to produce the Moloney *sav* cell line NM-sav (as discussed later in section 4.3B). In contrast, pNEO requires the insertion of transcriptional factors 5' to the *neo*^{r} gene for expression of this gene to occur in mammalian cells. In this study, these factors (transcription initiation site, promotor and enhancer sequences etc.) are supplied by a retroviral LTR. It is this vector that is used in the construction of the neomycin resistance selection vectors, described in the following section.

4.2 Construction of neomycin resistance selection vectors

A) Neomycin resistance selection vectors carrying the *gag* gene

1) The Kirsten *gag* expression vector pNEO Ki gag

The construction of pNEO Ki gag is shown in Figure 4.2A.ia and the methods used are described in section 2.8. The pNEO Ki gag plasmid DNA was digested with a range of restriction enzymes and the results from this restriction analysis are shown in Figure 4.2A.ib. The patterns of restriction fragments

Legend to figure 4.2A.ia. Construction of the neomycin resistance selection vector pNEO Ki *gag*. pNEO was digested with EcoRI and Hind III, dephosphorylated with CIAP and then the vector fragment (~5500 bp) was isolated. pUC Ki *gag*1 was digested with EcoRI and Hind III and the LTR *gag* LTR fragment (~3500 bp) was isolated. The two fragments were ligated to form pNEO Ki *gag*.

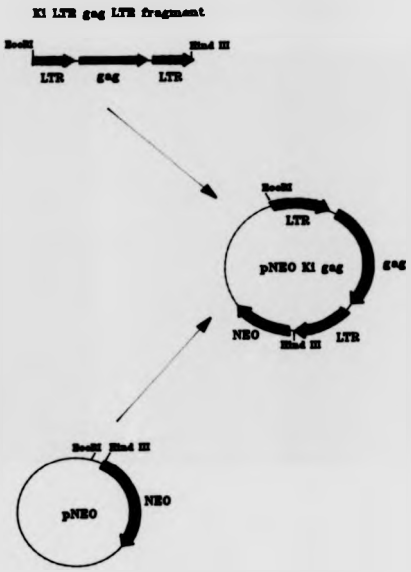
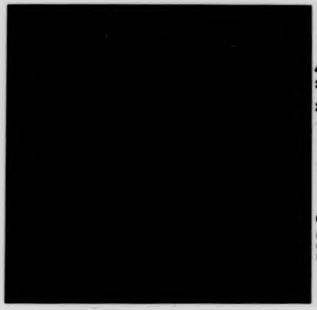


Figure 4.2A.ia. Construction of the neomycin resistance selection vector pNEO K1 gag



Legend to figure 4.2A.1b. Restriction map analysis of the expression vector pHRO Ki gag. Plasmid DNA was restricted with BamH I (e), Pst I (d), BamH I/Hind III/EcoR I (c), EcoR I (b) and EcoR I/Hind III (a). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with BamH I would result in fragments of 1.55 and 7.45 kbp. See Appendix D

are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. The LTR *gag* LTR fragment isolated from the expression vector pUC Ki gag contains ~350 bp of *pol* sequence (not shown in Figure 4.2A.ia) which is thought not to interfere with expression of the *gag* gene.

This vector is designed such that the LTR 3' to the *gag* gene will drive transcription of the *neo*^{r} gene. This use of a LTR to drive transcription of the *neo*^{r} gene has successfully been employed previously. In this instance, the complete Ki LTR (isolated from pUC Ki LTR AB) was placed 5' to the *neo*^{r} gene of pNEO to form the neomycin resistance selection vector pKiLTR-NEO. When transfected into the fibroblast cell line C3H10T½, this vector was shown to confer resistance to the antibiotic G418 (data not shown), thus it appears that this LTR contains the necessary transcription factors for the expression of the *neo*^{r} gene.

2) The Moloney *gag* expression vector pNEO Mo gag

The construction of pNEO Mo gag is shown in Figure 4.2A.iia and the methods used are described in section 2.8. The pNEO Mo gag plasmid DNA was digested with a range of restriction enzymes and the results from this restriction analysis are shown in Figure 4.2A.iib. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. Similar to above, the LTR *gag* LTR fragment isolated from pUC Mo gag contains some *pol* sequence (~700 bp) which is thought not to interfere with the expression of the *gag* gene and the LTR 3' to the *gag* gene is positioned to drive transcription of the *neo*^{r} gene. Furthermore, the complete Mo LTR (from pUC Mo LTR) was used to form pMoLTR-NEO, which also conferred

Legend to figure 4.2A.11a. Construction of the neomycin resistance selection vector pNEO Mo *gag*. pNEO was digested with EcoRI and Hind III, dephosphorylated with CIAP and then the vector fragment (~5500 bp) was isolated. pUC Mo *gag* was digested with EcoRI and Hind III. The LTR *gag* EcoRI/EcoRI fragment (~3100 bp) and the LTR EcoRI/Hind III fragment (~700 bp) were isolated. A three way ligation was performed with the above fragments to form pNEO Mo *gag*.

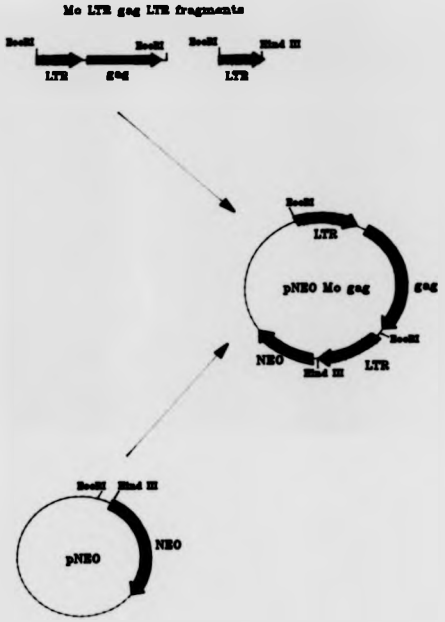
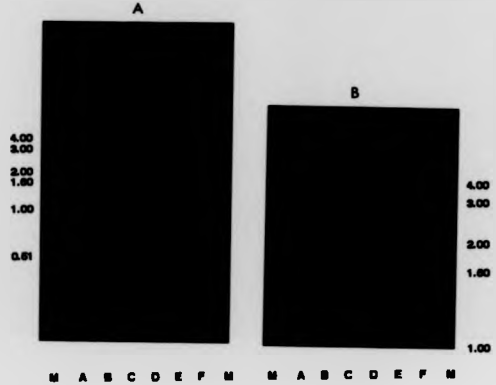


Figure 4.2A.iia. Construction of the neomycin resistance selection vector pNEO Mo gag



Legend to figure 4.2A.11b. Restriction map analysis of the neomycin resistance selection vector pMEX Mo gag. Plasmid DNA was restricted with Pst I (a), Bgl II (b), EcoR I/Hind III (c), EcoR I (d), Hind III (e) and Pst I/Bgl II (f). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with Bgl II would result in fragments of 2.10, 7.80 kbp. See Appendix D.

resistance to G418 (data not shown). Accordingly, the Mo LTR can also drive transcription of the *neo*^{r} gene.

B) Mycophenolic resistance selection vectors carrying the *sav* gene

1) The Kirsten *sav* expression vector pMRO Ki sav

The construction of pMRO Ki sav is shown in Figure 4.2B.ia and the methods used are described in section 2.8. The pMRO Ki sav plasmid DNA was digested with a range of restriction enzymes and the results from this restriction analysis are shown in Figure 4.2B.ib. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. The LTR *sav* LTR fragment isolated from the expression vector pUC Ki sav also contains a small amount of *pol* sequence (not shown in Figure 4.2B.ia). Additionally, as described for pUC Ki sav, the splice donor and acceptor sites of the MLV genome are utilised for the expression *sav* gene products. This vector is also designed such that the LTR 3' to the *sav* gene will drive transcription of the *neo*^{r} gene.

Legend to figure 4.2B.ia. Construction of the neomycin resistance selection vector pNEO K1 *env*. pNEO was digested with EcoRI and Hind III, dephosphorylated with CIAP and then the vector fragment (~5500 bp) was isolated. pUC K1 *env* was digested with EcoRI and Hind III and the LTR *env* LTR fragment (~3700 bp) was isolated. The two fragments were then ligated to form pNEO K1 *env*.

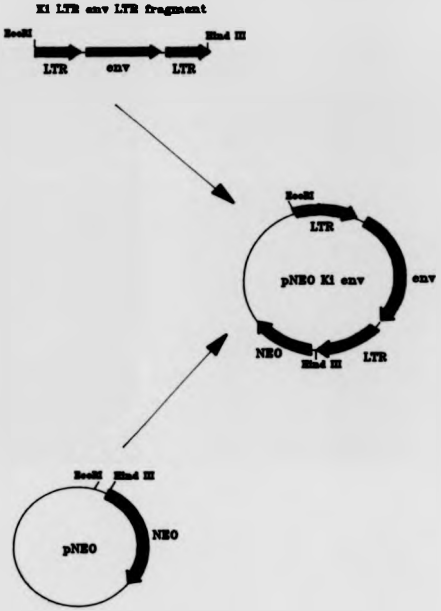
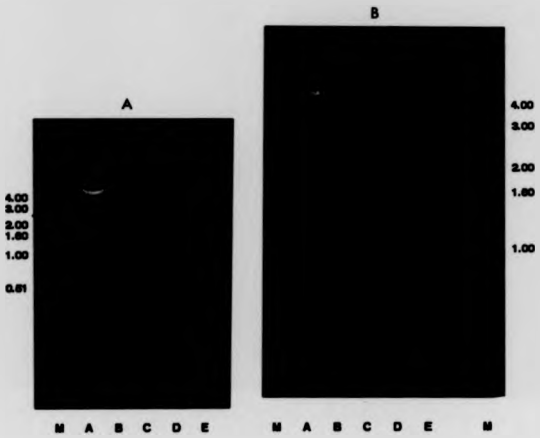


Figure 4.2B.ia. Construction of the neomycin resistance selection vector pNEO Ki env



Legend to figure 4.2B.1b. Restriction map analysis of the neomycin resistance selection vector pNEO Ki env. Plasmid DNA was restricted with BamH I (a), EcoR I/Hind III (b), Pst I (c), Kpn I (d), EcoR I (e) and H/A (f). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with BamH I would result in fragments of 0.50, 0.75, 1.55, 1.80 and 4.70 kbp. See Appendix D

4.3 Generation of cell lines expressing the neomycin resistance selectable marker and the MLV gene of interest

All transfected cell lines are tabulated in Appendix B for easy reference.

A) The use of pNEO Ki gag, pNEO Mo gag and pNEO Ki env to generate neomycin resistant cell lines expressing the MLV gene of interest.

The expression vectors pNEO Ki gag, pNEO Mo gag and pNEO Ki env were transfected into the *ras* transformed embryo fibroblast cell line C3H201 as described in section 2.9F. The DNA used for each transfection was of large scale plasmid preparation quality and had been isolated on a caesium chloride gradient. Control transfections were carried out to ensure that the transfection procedure was working efficiently. This was necessary as it had not been previously shown that vectors designed as described above would indeed function. A lack of neomycin resistant colonies could therefore be due to a failure of the LTR to drive transcription of the *neo*^{r} gene or an unsuccessful transfection procedure. The control involved transfecting the C3H201 cell line with the neomycin resistance vector pSV2neo which had previously been shown to confer resistance to the antibiotic G418 in this cell line (A. G. Morris, personal communication). After transfection with the selection vectors the cells were cultured for a further 3-4 days before placing them under selection conditions (as described in section 2.9G). The selected cell lines after transfection with pNEO Ki gag, pNEO Mo gag and pNEO Ki env are referred to as NK-gag1, NK-gag and NK-env, respectively. pKiLTR-NEO and pMoLTR-NEO were also transfected into the C3H201 cell line and the resultant neomycin resistant cell lines (both referred to as NC3H201) were used as the appropriate negative controls to NK-gag1, NK-gag and NK-env in the Tc assays and tumour growth studies discussed in the last section of this chapter.

B) Generation of neomycin resistant cell lines expressing the MLV genes of interest by co-transfection of the MLV gene with a neomycin resistance selection vector

The expression vector pUC Ki gag was co-transfected with pSV2neo into the C3H201 cell line to produce the neomycin resistant cell line, NK-gag2, after selection with G418 (section 2.9G). Similarly, C3H201 cells were co-transfected with pUC Mo env and pSV2neo to produce the cell line NM-env. As described above, the DNA preparations used for the transfections were of the highest quality and a control transfection with pSV2neo alone was carried out concurrently to monitor the efficiency of the transfection procedure. Furthermore, pUC Ki gag and pUC Mo env were linearised prior to their use in a transfection by cutting within the pUC13 sequence with a suitable restriction enzyme.

4.4 Analysis of mRNA prepared from neomycin selected cells

In this study, the dot-blot hybridisation procedure described in section 2.10B is used to analysis the mRNA prepared from the selected cell lines for expression of the transfected genes of interest. This method is based on the ability of mRNA to hybridize specifically a radiolabelled DNA sequence to its complementary mRNA sequence. The radiolabelled DNA sequence (also referred to as DNA probe) was prepared as described in section 2.8I. To minimise non-specific binding of the DNA probe, only sequences complementary to the mRNA of interest were used and no flanking plasmid sequences were included. The mRNA from the neomycin selected cells was prepared as described in section 2.10A. If mRNA was not to be used immediately, it was stored in a precipitated form in ethanol at -70°C. All transfected cell lines are tabulated in Appendix B.

A) Analysis of *gag* specific mRNA

i) Examination of mRNA from the putative Kirsten *gag* expressing cell lines NK-gag1 and NK-gag2

Figure 4.4A.i shows the results from the dot-blot hybridisation analysis of mRNA prepared from NK-gag1 and NK-gag2. The appropriate positive and negative control cell lines used in this analysis were KC3H and C3H201, respectively. As already mentioned (section 3.3A.i), the *ras* transformed cell line KC3H expresses all the Ki MLV polypeptides and thus is positive for Ki *gag* specific mRNA. The C3H201 cell line, although *ras* transformed, expresses no MLV polypeptides and thus is negative for *gag* specific mRNA. C3H201 is the appropriate negative control for this analysis as all the transfected cell lines were derived from this line. However C3H10T½ (3.3A.i) are also shown in this figure as an alternative negative control for Ki *gag* specific hybridisation. For each RNA sample, 5 and 10 μg were dotted onto the nitrocellulose filter.

The DNA probe used for this hybridisation analysis was prepared from a Pvu II/ Sac I fragment of the Ki MLV clone cP$_2$ (section 2.1). This DNA probe, which contains *gag* coding sequence only, shows specific binding to the KC3H mRNA with no significant hybridisation observed with the negative control cell lines C3H201 and C3H10T½. Positive hybridisation with the cell lines NK-gag1 and NK-gag2 was seen, indicating the presence of Ki *gag* specific mRNA in these transfected cell lines.

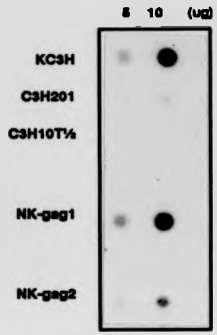


Figure 4.4A.i. Examination of mRNA from putative Kirsten gag expressing cell lines NK-gag1 and NK-gag2. The mRNA was analysed by dot blot hybridisation as described in section 2.10B. The filter was washed at high stringency (0.1x SSC, 0.1% SDS, 65°C) and the autoradiograph was exposed for 24 hours at -70°C using a Du Pont Cronex intensifying screen.

ii) Examination of RNA from the putative Moloney *gag* expressing cell line MM-gag

Figure 4.4A.ii shows the results from the dot-blot hybridisation analysis of mRNA prepared from MM-gag; the total selected population (MM-gag) and the 6 cloned cell lines derived from this population (A - F). The cell lines A - F were derived from MM-gag and were maintained in selective medium for 3 weeks before use (for the cloning procedure see section 2.9I). The appropriate positive and negative control cell lines used in this analysis were MoSVC3H and C3H201, respectively. It was noted in section 3.3A.ii that the mos transformed cell line MoSVC3H expresses all Mo MLV polypeptides, and therefore is positive for Mo *gag* specific mRNA. As above, C3H201 are negative for Mo *gag* specific mRNA. For each RNA sample, 5, 10 and 20 μg were dotted onto the nitrocellulose filter.

The DNA probe used for this hybridisation analysis was prepared from a Pvu I fragment of Mo MLV. This fragment, prepared from the vector pZAP (section 2.1) contains the majority of the *gag* coding region and no other sequence. Specific hybridisation with the mRNA of MoSVC3H was observed with no detectable non-specific binding with C3H201. The total selected population of MM-gag shows significant hybridisation with the radiolabelled probe, thus indicating the presence of Mo *gag* specific mRNA in these cells. However, the cells cloned from this line are variable in their level of expression of Mo *gag* specific mRNA, with all clones except A showing low levels of expression.

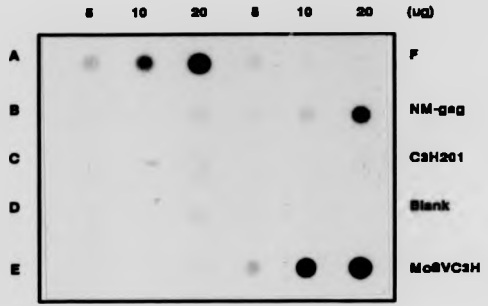


Figure 4.4A.ii. Examination of mRNA from putative Moloney *gag* expressing cell line NM-gag. The mRNA was analysed by dot blot hybridisation as described in section 2.10B. The filter was washed at high stringency (0.1x SSC, 0.1% SDS, 65°C) and the autoradiograph was exposed for 18 hours at -70°C using a Du Pont Cronex intensifying screen. The total selected population NM-gag and 6 cloned cell lines derived from this population (A-F) were analysed.

B) Analysis of *env* specific RNA

i) Examination of RNA from the putative Kirsten *env* expressing cell line NK-env

Figure 4.4B.i shows the results from the dot-blot hybridisation analysis of mRNA prepared from NK-env. The appropriate positive and negative control cell lines used in this analysis were KC3H and C3H201, respectively. KC3H, as discussed previously, expresses all the Ki MLV polypeptides and thus Ki *env* specific mRNA is found in these cells. C3H201 do not express Ki MLV polypeptides and so contain no Ki *gag* specific mRNA. The DNA probe used for this hybridisation analysis was prepared from the BamH I/ Pst I fragment of the Ki clone cPi which contains *env* coding sequence (section 2.1).

The probe was found to hybridise with the mRNA prepared from the positive control cell line KC3H and the selected cell line NK-env. This hybridisation is thought to be specific as no significant binding was observed with this probe to the negative control cell line C3H201 mRNA. Hence, it would appear from this analysis that the selected cell line NK-env expresses Ki *env* specific mRNA.

ii) Examination of RNA from the putative Moloney *env* expressing cell line MK-env

Figure 4.4B.ii shows the results from the dot-blot hybridisation analysis of mRNA prepared from MK-env. Similar to section 4.4A.ii, the control cell lines used were MoSVC3H and C3H201; the former being positive for Mo *env* specific mRNA expression and the latter negative. For each RNA sample, 1, 5, 10 and 20 μg were dotted onto the nitrocellulose filter. The DNA probe used for this hybridisation analysis was prepared from an Xba I/ Pvu II fragment containing the *env* coding region. This probe was found to hybridise with the mRNA prepared from MoSVC3H and MK-env. Again no

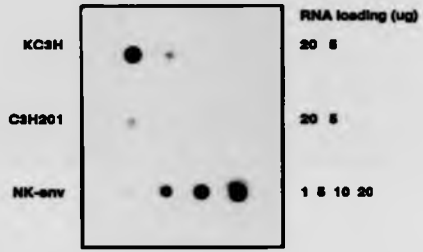


Figure 4.4B.i. Examination of mRNA from putative Kirsten *env* expressing cell line NK-env. The mRNA was analysed by dot blot hybridisation as described in section 2.10B. The filter was washed at high stringency (0.1x SSC, 0.1% SDS, 65°C) and the autoradiograph was exposed for 38 hours at -70°C using a Du Pont Cronex intensifying screen.

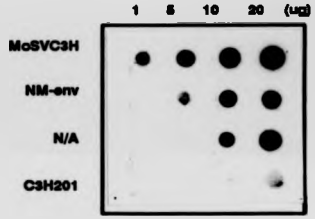


Figure 4.4B.ii. Examination of mRNA from the putative Moloney *env* expressing cell line NM-env. The mRNA was analysed by dot blot hybridisation as described in section 2.10B. The filter was washed at high stringency (0.1x SSC, 0.1% SDS, 65°C) and the autoradiograph was exposed for 60 hours at -70°C using a Du Pont Cronex intensifying screen. The NM-env samples were incorrectly loaded (1, 5, 20, 10).

significant binding was observed with mRNA prepared from C3H201, thus it is proposed that NK-env expresses Mo *env* specific mRNA.

4.5 Analysis of polypeptides produced by mammalian selected cells

The analysis of polypeptides produced by the selected cell lines was performed by either radioimmunoprecipitation followed by one-dimensional SDS-PAGE or SDS-PAGE followed by western blotting (section 2.11). The radiolabelled polypeptides separated by electrophoresis were identified by autoradiography. The proteins western blotted onto nitrocellulose were visualised with the appropriate anti-gag or anti-env antibody using biotin-streptavidin peroxidase colour labelling. All transfected cell lines are tabulated in Appendix B.

A) Analysis of *gag* polypeptides

i) Examination of polypeptides produced by the putative Kirsten *gag* expressing cell lines NK-gag1 and NK-gag2

Figure 4.5A.ia shows the anti-Ki-gag immunoprecipitated polypeptides of NK-gag1 separated by SDS-PAGE. The positive and negative control cell lines used were KC3H and C3H201, respectively. It can be seen from track A that the anti-Ki-gag antibody efficiently immunoprecipitates the p30 *gag* protein in the positive control cell line, however the precursor protein to p30 ($pr65^{gag}$) is not present in this track. This may be due to either an inability of the antibody to immunoprecipitate $pr65^{gag}$ efficiently from the KC3H cell lysate; or the too rapid processing of this precursor protein such that it escapes detection. In the case of the former, this is very significant because, as discussed in detail before, the transfected cell lines NK-gag1 and NK-gag2 are unable to cleave $pr65^{gag}$ into the smaller *gag* proteins.

Attempts were next made to use this anti-Ki-gag antibody in western immunodetection analysis of the polypeptides produced by NK-gag1 and NK-gag2. Figure 4.5A.ib shows the results from this analysis. As above, the

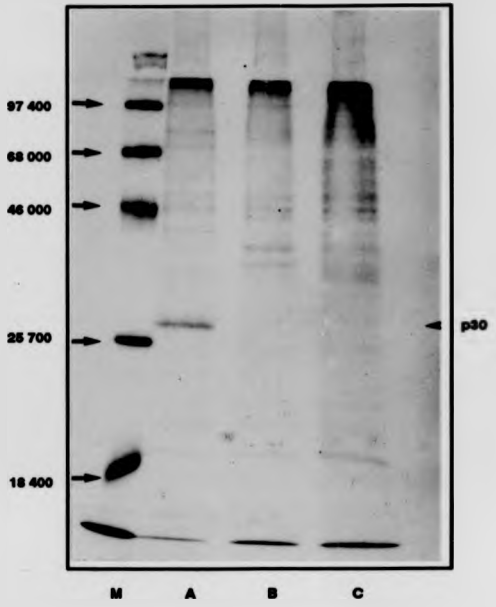


Figure 4.5A.ia. Examination of the polypeptides produced by the putative Kirsten *gag* expressing cell lines NK-gag1 by radioimmunoprecipitation. Cells were labelled with ^{35}S-methionine and the labelled proteins were immunoprecipitated with anti-Ki-gag serum and analysed by SDS-PAGE and fluorography using a pre-flashed x-ray film.

Track M: molecular weight markers (Daltons)
A: KC3H
B: C3H201
C: NK-gag1

The position of the p30 protein is indicated.

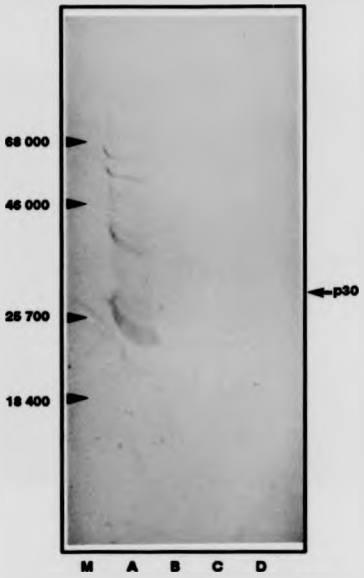


Figure 4.5A.ib. Examination of the polypeptides produced by the putative Kirsten *gag* expressing cell lines NK-gag1 and NK-gag2 by western immunodetection. Samples were separated by SDS-PAGE, western blotted and Ki *gag* polypeptides visualised with peroxidase labelled anti-Ki-gag serum.

Track M: molecular weight markers (Daltons)
A: KC3H
B: C3H201
C: NK-gag1
D: NK-gag2

The position of the p30 protein is indicated.

positive and negative control cell lines used were KC3H and C3H201, respectively. It can be seen from track A that the anti-Ki-gag antibody efficiently detects the p30 *gag* protein expressed by KC3H. It also detects many other polypeptides, but does not react with a protein band that is of the correct m.wt. to represent the precursor polypeptide $pr65^{gag}$. Again, this may be due to either an inability of the antibody to detect the precursor protein; or the too rapid processing of $pr65^{gag}$. This lack of detection of $pr65^{gag}$ in the KC3H lysate complicates the interpretation of the results from the transfected cell lines HK-gag1 and HK-gag2 (tracks C and D respectively). The absence of the precursor protein in tracks C and D could be due to the inability of the antibody to detect $pr65^{gag}$ present on the nitrocellulose even when present in large amounts. Alternatively, the transfected cell lines HK-gag1 and HK-gag2 may not express $pr65^{gag}$ or may express it at a level that is undetectable by western immunodetection. However, the mRNA analysis of these cell lines (discussed in section 4.4) does suggest that the expression of $pr65^{gag}$ does occur.

ii) Examination of polypeptides produced by the putative Moloney *gag* expressing cell line HK-gag

Figure 4.5A.iia shows the anti-Mo-gag immunoprecipitated polypeptides of HK-gag separated by SDS-PAGE. The positive control cell lines used for this analysis were MoSVC3H (as described in section 4.3A.ii) and MoLV201. MoLV201 was derived from C3H201 by transfection of the parent cell line with the vector pZAP which contains the proviral DNA of Mo MLV (see Appendix B). The negative control cells used for this analysis were C3H201. It can be seen from track A and B that when MoSVC3H and MoLV201 cells (respectively) are immunoprecipitated with this antibody, p30 is precipitated efficiently as observed above with the anti-ki-gag antibody, but in addition $pr65^{gag}$ is also precipitated. Furthermore, it is later

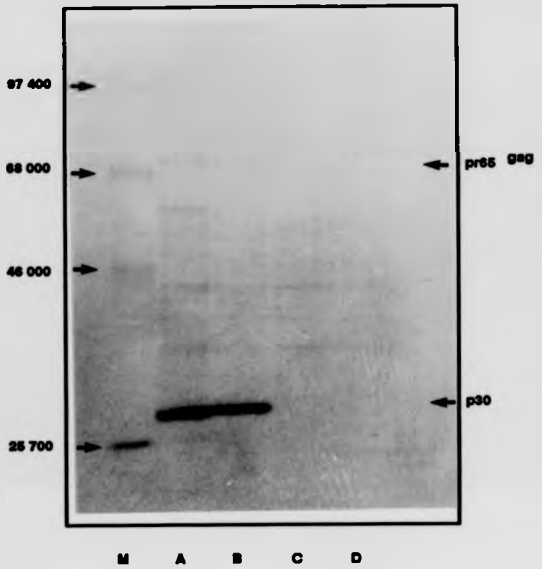


Figure 4.5A.iia. Examination of the polypeptides produced by the putative Moloney *gag* expressing cell lines NM-gag by radioimmunoprecipitation. Cells were labelled with ^{35}S-methionine and the labelled proteins were immunoprecipitated with anti-Mo-gag serum and analysed by SDS-PAGE and fluorography using a pre-flashed x-ray film.

Track M: molecular weight markers (Daltons)
A: MoSVC1M
B: MoLV201
C: C3H201
D: NM-gag

The positions of the p30 and pr65^{gag} are indicated.

shown (in chapter 5) that precursor proteins $pr65^{gag}$ and $pr75^{gag}$ transcribed and translated *in vitro* are immunoprecipitated with the anti-Mo-gag antibody. Thus this antibody under certain conditions can be used to detect the precursor polypeptides. No $pr65^{gag}$ is detectable in track D suggesting that either no $pr65^{gag}$ is produced in these transfected cells or that the amount expressed is insufficient to be detected by this antibody via immunoprecipitation.

In a manner similar to that described above, the cells were then analysed by western immunodetection using the same anti-Mo-gag antibody. Figure 4.5A.iib shows the results from this analysis. MoSVC3H were used as the positive control cell line and both C3H2O1 and C3H10T½ were used as negative controls. Examination of the MoSVC3H cell line in track A shows that the anti-Mo-gag antibody can detect p30 (and other proteins) by this method although no $pr65^{gag}$ was identified in this case. Examination of tracks F and G (cf. negative control tracks) indicates that the transfected line MH-gag expresses the precursor polypeptide $pr65^{gag}$, as a protein with the predicted molecular weight of $pr65^{gag}$ is observed. Although initially these results appear to contradict the immunoprecipitation results described above, it is proposed that they may be reconciled with the following considerations. First, the anti-Mo-gag antibody may detect $pr65^{gag}$ more efficiently by western immunodetection than by immunoprecipitation. Secondly, the amount of $pr65^{gag}$ present in the MoSVC3H cell line appears very variable (from lysate-to-lysate) and consequently is not a reliable positive control. It is thus suggested that the MoSVC3H and MoLV2O1 lysates of figure 4.5A.iia (track A and B) contained a greater amount of the precursor protein $pr65^{gag}$, thus it was possible to detect it even by the less efficient method of immunoprecipitation. In contrast, the amount of $pr65^{gag}$ expressed by the transfected cells was insufficient to be

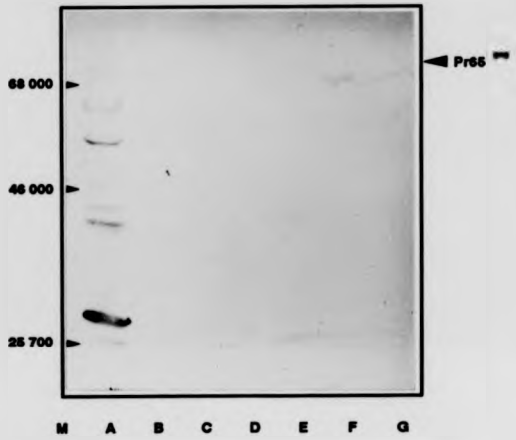


Figure 4.2A.iib. Examination of polypeptides produced by Moloney *gag* expressing cell line NM-gag by western immunodetection. Samples were separated by SDS-PAGE, western blotted and Mo *gag* polypeptides visualised with peroxidase labelled anti-Mo-gag serum.

Track M: molecular weight markers (Daltons)
A: MoSVC3H (50 µl cell lysate)
B: C3H201 (50 µl cell lysate)
C: C3H201 (20 µl cell lysate)
D: C3H10T½ (20 µl cell lysate)
E: C3H10T½ (50 µl cell lysate)
F: NM-gag (50 µl cell lysate)
G: NM-gag (20 µl cell lysate)

The positions of the proteins p30 $Pr65^{gag}$ are indicated.

detected by this method. Alternatively, the MoSVC3H lysate prepared for figure 4.5A.iib contained undetectable levels of $pr65^{gag}$ (even by western immunodetection) whereas the precursor polypeptide expressed by MK-gag could be detected by this method.

B) Analysis of *env* polypeptides

i) Examination of polypeptides produced by the putative Kirsten *env* expressing cell line MK-env

Figure 4.5B.i shows the results from the western immunodetection analysis of the polypeptides produced by MK-env with the anti-Ki-env antibody. The appropriate positive and negative control cell lines used for this analysis were KC3H and C3H201, respectively (as for section 4.5A.i). It appears from these results that a polypeptide endogenous to the C3H201 cell line runs at the same position as gp70 and is detected with the anti-Ki-env antibody by western immunodetection (tracks A and B). Although it was reported by the isolator of the C3H10T½ cell line that it contains no 'overt' endogenous transforming murine leukaemia or sarcoma viruses (see ATCC catalogue), it now appears that these cells may contain an endogenous provirus (Weiss *et al.*, 1985) and that the *env* gene of this provirus may be expressed (at a low level) in the derivative cell line C3H201 perhaps because of the *ras* transformation (C3H10T½ not examined for gp70 expression). As discussed in detail elsewhere (Weiss *et al.*, 1985) many vertebrate species carry genetically transmitted retroviral genomes and the viral glycoprotein coded by the *env* gene of these genomes is frequently expressed to varying degrees at the cell surface. It can be seen by comparison with the KC3H tracks (C-E) that the level of expression of the putative gp70 polypeptide in the C3H201 cell line and its derivative MK-env is low. This low level expression of an endogenous gp70 protein in the C3H201 cell line was not detected during the mRNA analysis of these cells with a Ki *env* specific radiolabelled probe

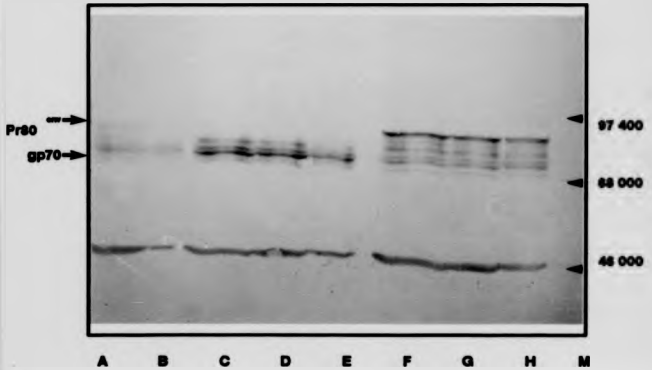


Figure 4.3B.i. Examination of the polypeptides produced by the putative Kirsten *env* expressing cell line NK-env by western immunodetection. Samples were separated by SDS-PAGE, western blotted and Ki *env* polypeptides visualised with peroxidase labelled anti-Ki-env serum.

Track M: molecular weight markers (Daltons)
A: C3H201 (30 µl cell lysate)
B: C3H201 (20 µl cell lysate)
C: KC3H (30 µl cell lysate)
D: KC3H (30 µl cell lysate)
E: KC3H (20 µl cell lysate)
F: NK-env (30 µl cell lysate)
G: NK-env (30 µl cell lysate)
H: NK-env (20 µl cell lysate)

The positions of gp70 and the putative $Pr80^{env}$ protein are indicated.

(4.4B.i). This may reflect a greater difference at the nucleic acid level than the protein level between the putative endogenous gp70 and exogenous polypeptide. Alternatively, this protein may be a normal cellular product in the C3H201 cell line that unfortunately reacts with this antibody and runs at the same position as gp70 on a one-dimensional gel. There are also additional minor bands present in the KC3H tracks which similarly may be cellular proteins that are detected by the anti-Ki-*env* antibody, however it is suggested that these proteins may be intermediate cleavage products of $Pr80^{env}$.

Examination of tracks F-H in figure 4.5B.i show that the NK-*env* cell line expresses a number of polypeptides that are detected with the anti-Ki-*env* antibody. In contrast to KC3H, however, the major polypeptide is not gp70, but a protein of a higher molecular weight (approx. 84 kD). In section 3.3B.i it was predicted, by comparison with similar expression vectors, that the Ki *env* precursor protein $Pr80^{env}$ produced by the Ki *env* expression vectors would be cleaved into gp70 and p15E. From the results presented here it is proposed that this is not the case; it is suggested that this 84 kD polypeptide is the uncleaved precursor protein.

In cells expressing Ki and Mo-MLV, the primary translation product of the *env* gene, $Pr80^{env}$, is synthesized with a leader peptide (codon 1 to 33 of the *env* gene) that is removed by cellular enzymes in the rough endoplasmic reticulum accompanying glycosylation of the *env* precursor (Dickson *et al.*, 1982). Next, the *env* precursor is cleaved in the Golgi to the glycoprotein, gp70 and p15E (Fitting and Kabat, 1982). Subsequent to this cleavage, some of the p15E molecules are then cleaved to p12 (Dickson *et al.*, 1982). It was reported by Flyer *et al.* (1983), as discussed before, that the first and second cleavage of $Pr80^{env}$ occurred successfully in the cells

expressing their constructs. In contrast, it appears that the process of cleaving the precursor protein is impaired in the MK-env cell line, although the minor products in tracks F-H suggest that some cleavage does occur (some of these smaller polypeptides as described above are also detected in the KC3H tracks (C-H)). Thus it is proposed that the transfected cell line MK-env expresses the *env* precursor protein (as suggested by RNA analysis), however the processing of this protein is defective.

ii) Examination of polypeptides produced by the putative Moloney *env* expressing cell line MK-env

Figure 4.5B.ii shows the results from the western immunodetection analysis of the polypeptides produced by MK-env with an anti-Mo-env antibody. The positive and negative control cell lines used for this analysis were MoSVC3H and C3H201, respectively (as for section 4.5A.ii). From examination of track A it can be seen that the anti-Mo-env antibody detects a polypeptide of ~70 KD in the MoSVC3H lysate and this protein is proposed to be gp70. In a manner similar to that described in section 4.5B.i, a polypeptide of this size is also detected in the C3H201 cell line (track B) with this antibody, thus supporting the proposal that this cell line contains an endogenous provirus. Again, this low level expression of an endogenous gp70 protein was not detected during mRNA analysis of these cells with an Mo *env* specific radiolabelled probe (4.4B.ii). But, as suggested before, this may reflect a greater difference between the putative endogenous gp70 and exogenous polypeptide at the nucleic acid level than at the protein level.

Examination of track C (cf. track B) suggests that the transfected cell line MK-env is expressing the *env* precursor protein $Pr80^{env}$. Furthermore,

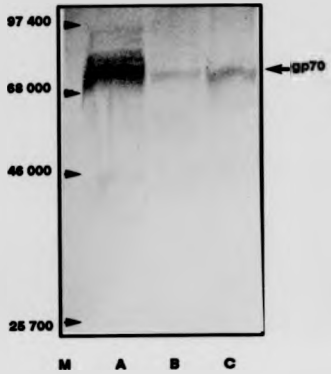


Figure 4.5B.ii. Examination of the polypeptides produced by the putative Moloney *env* expressing cell line NM-env. Samples were separated by SDS-PAGE, western blotted and Mo *env* polypeptides visualised with peroxidase labelled anti-Mo-env serum.

Track M: molecular weight markers (Daltons)
A: MoSVC3H
B: C3H2O1
C: NM-env

The position of the gp70 protein is indicated.

it appears that this precursor is successfully cleaved as the cleavage product gp70 not $Pr80^{env}$ (as for MK-env) is detected with the anti-Mo-env antibody.

4.6 The use of neomycin resistant cells to investigate the immune response to specific antigens on tumour cells

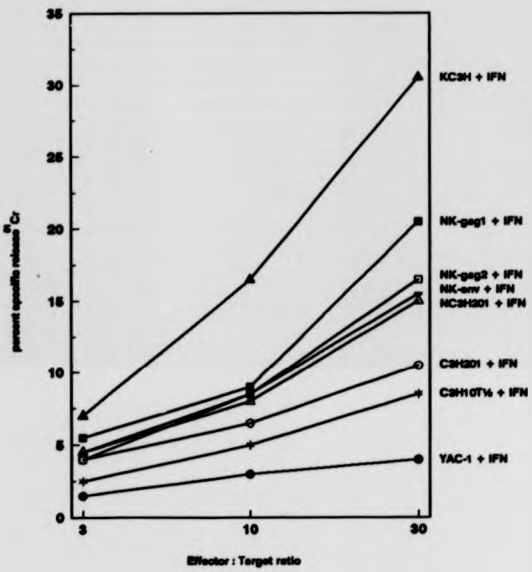
Two methods of investigation were used to examine the immune response to the neomycin resistant cell lines described in the previous section. First, the Tc response to these cells was examined in an *in vitro* chromium release assay as described in section 2.12B. Second, the *in vivo* growth of these neomycin resistant *ras* transformed cell lines was observed as described in section 2.12C. For each study the cells used were of approximately the same passage number. This is particularly significant in the tumour growth studies as it has been shown by other group members that the tumourigenicity of the C3H201 cell line greatly increases with passage number (R. Darley, personal communication). All transfected cell lines are tabulated in Appendix B.

A) Examination of the Tc response to specific antigens on tumour cells

i) Analysis of the Tc response to tumour cells expressing Kirsten specific MLV antigens

Figure 4.6A.is shown the susceptibility of the cell lines NK-gag1, NK-gag2 and NK-env to lysis by Ki-MSV/MLV-specific Tc (all cell lines were IFN-γ treated). It can be seen that the EC3H cell line is highly susceptible to lysis by these Tc whereas the C3H10T½, C3H201 and Yac-1 cell lines were not killed to a significant amount. This confirms that a Ki-MSV/MLV-specific Tc population has been generated and that no detectable natural killer cell activity is present in this population. The NK-gag1 cell line, which makes more *gag*-specific mRNA than NK-gag2, was clearly susceptible to lysis, however the NK-gag2 and NK-env lines were only slightly more susceptible than the control line EC3H201, which as discussed before, was derived from C3H201 cells by transfection with the neomycin resistance vector pKiLTR-NEO. It is clear from these results that the *gag* expressing NK-gag1

Figure 4.6A.1a. The susceptibility of the cell lines NK-gag1, NK-gag2 and NK-env to lysis by KI-MSV/MLV-specific cytotoxic T lymphocytes

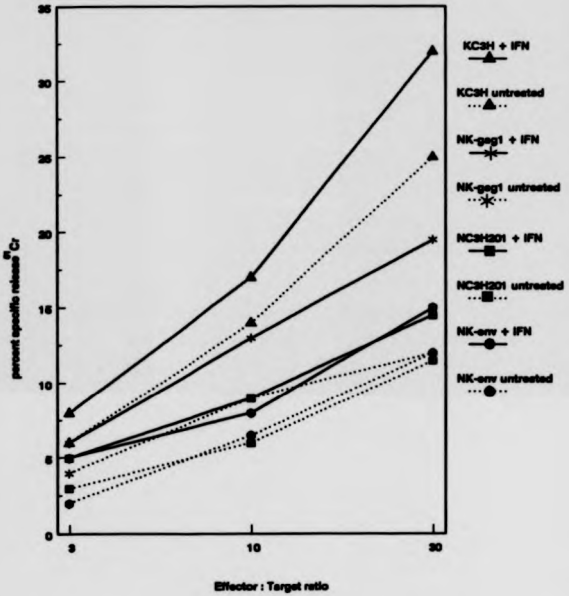


The susceptibility of the cells to lysis by KI-MLV/MSV-specific cytotoxic T lymphocytes was determined as described in section 2.12.B. These results are the average of 6 replicates from the same experiment (SE ≤ 1.6 % lysis). This experiment was repeated twice and these results are representative.

cell line is an efficient target for Ki-MSV/MLV-specific Tc (although not as efficient as the MC3H cells), indicating that this transfected cell line displays antigen that the Ki-MSV/MLV-specific Tc can recognise. However the increased killing of MC3H201 over the parental line C3H201 makes the interpretation of these results more difficult. It appears that expression of the *neo*^{r} gene in the MC3H201 cell line increases killing by the Ki-MSV/MLV-specific Tc. It is possible that either the *neo*^{r} gene product is recognised by the effector population or that expression of this gene induces the expression of other proteins which in turn are recognised by these cells. Alternatively, the selection of these transfected cells in G418 may result in a biased population of cells due to preferential growth of some selected cells over others although all the cells express the *neo*^{r} gene. For instance, Paludan *et al.* (1989) describe a situation where graduated resistance to G418 leads to differential selection of cultured mammalian cells expressing the *neo*^{r} gene. They found that cells expressing high levels of *neo*^{r} grew efficiently, whereas low expressors were often killed thus resulting in a cell line unrepresentative of the selected population. In a similar manner the MC3H201 cells may be biased representatives of the potential selected population. Likewise, it is difficult to determine the significance of the increased killing of the MK-env cells. A detailed examination of this problem exceeds the boundaries of this study, however it appears from these results that the use of neomycin selection is not suitable for the investigations of this work.

The effect of recombinant IFN-γ treatment on the susceptibility of the cell lines MK-gag1 and MK-env to lysis by Ki-MSV/MLV-specific Tc is shown in figure 4.6A.ib. It can be seen that IFN-γ treatment of MC3H and MK-gag1 cells greatly increases the susceptibility of these cells to lysis by Ki-MSV/MLV-specific Tc. However, the susceptibility of the cell lines MK-env

Figure 4.6A.Ib. The effect of IFN on the susceptibility of the cell lines NK-gag1 and NK-env to lysis by KI-MSV/MLV specific cytotoxic T lymphocytes



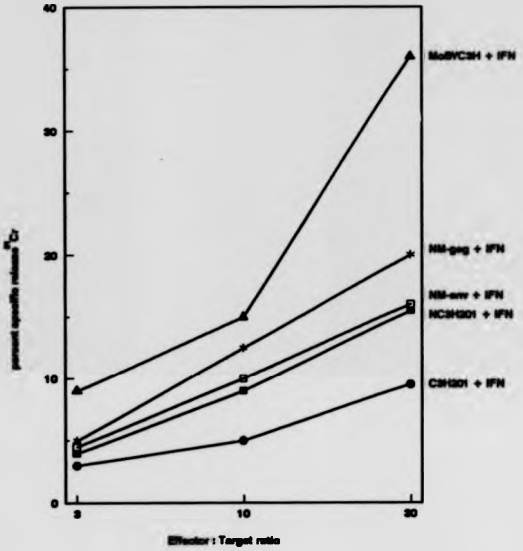
The susceptibility of the cells to lysis by KI-MLV/MSV-specific cytotoxic T lymphocytes was determined as described in section 2.12.B. These results are the average of 5 replicates from the same experiment (SE ≤ 1.0 % lysis). This experiment was repeated twice and these results are representative.

and NC3H201 to lysis by this Tc population is seemingly less affected by IFN-γ treatment, although an increase is observed. As discussed in the introduction, it is known that virus-specific Tc generally recognise viral antigen in association with class I MHC on the surface of target cells and that IFN-γ augments this class I MHC antigen expression on fibroblasts. It is therefore proposed that the significant increase in susceptibility observed for NC3H and NK-gag1 is due to an increase in class I MHC antigen expression on the cells after IFN-γ treatment. Again, both the NC3H201 and the NK-env cells were more susceptible to killing than the C3H201 cells (data not shown). As discussed above, no conclusions can be drawn due to insufficient information.

ii) Analysis of the Tc responses to tumour cells expressing Moloney specific MLV antigens

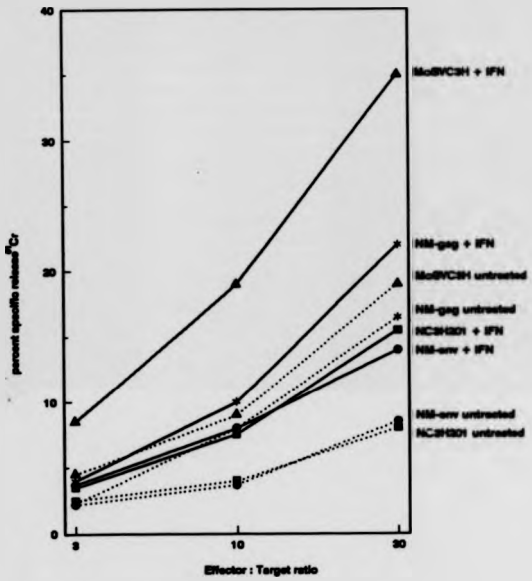
Figure 4.6A.iia shows the susceptibility of the cell lines NK-gag and NK-env to lysis by Mo-MSV/MLV-specific Tc (all cell lines were IFN-γ treated). The MoSVC3H cells are shown to be highly susceptible to lysis by these Tc, in contrast to the C3H201 cells which show a very low level of susceptibility. Similar to the above NK-gag1 cell line, the NK-gag cells show a significant amount of lysis by the Mo-MSV/MLV-specific Tc. Furthermore, the control cell line NC3H201 is again more susceptible than its parental line C3H201 (in this case, NC3H201 expresses pMoLTR-NEO). Figure 4.6A.iib shows the effect of recombinant IFN-γ treatment on the susceptibility of the cell lines NK-gag and NK-env to lysis by Mo-MSV/MLV-specific Tc. It appears that all the cell lines shown here (including the NC3H201) display increased susceptibility to Mo-MSV/MLV-specific lysis after IFN treatment. It is proposed that this increase in killing is due to an increase in class I MHC antigen expression as described above. It can be seen from figure 4.6A.iic that there is no significant killing of the cell

Figure 4.6A.IIa. The susceptibility of the cell lines NM-gag and NM-env to lysis by Mo-MSV/MLV-specific cytotoxic T lymphocytes



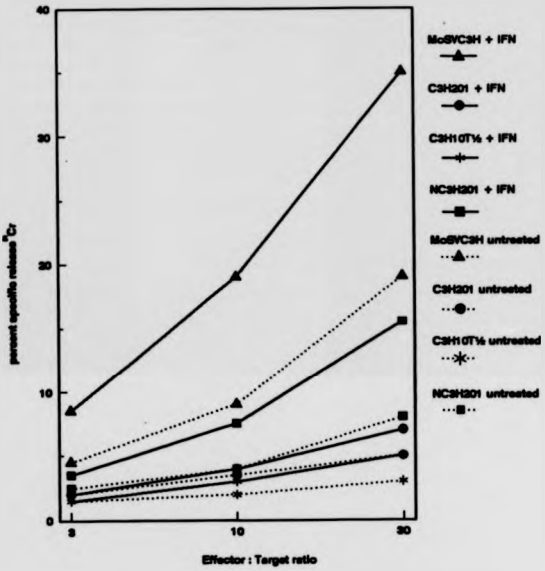
The susceptibility of the cells to lysis by Mo-MLV/MSV specific cytotoxic T lymphocytes was determined as described in section 2.13.B. These results are the average of 6 replicates from the same experiment (SE ≤ 1.2 % lysis). This experiment was repeated twice and these results are representative.

Figure 4.6A.IIb. The effect of IFN on the susceptibility of the cell lines NM-gag and NM-env to lysis by Mo-MSV/MLV specific cytotoxic T lymphocytes



The susceptibility of the cells to lysis by Mo-MLV/MSV specific cytotoxic T lymphocytes was determined as described in section 2.12.B. These results are the average of 6 replicates from the same experiment (SE ≤ 0.6). This experiment was repeated twice and these results are representative.

Figure 4.6A.IIc. The effect of IFN on the susceptibility of the cell line NC3H201 to lysis by Mo-MSV/MLV specific cytotoxic T lymphocytes



The susceptibility of the cells to lysis by Mo-MSV/MLV-specific cytotoxic T lymphocytes was determined as described in section 2.12.B. These results are the average of 6 replicates from the same experiment (SE ≤ 1.8). This experiment was repeated twice and these results are representative.

lines C3H10T½ and C3H2O1 and no notable effect by IFN (although a slight increase does occur). Thus analogous to the Kirsten system described above, it appears that expression of the *neo*^{r} gene in C3H2O1 cells affects the recognition of these cells by the Mo-MSV/MLV-specific Tc. Therefore again it is suggested that the use of neomycin selection is not suitable for the investigations of this work.

These results initially appear to contradict those of Flyer *et al.* (1983); they report no recognition by Mo-MSV/MLV-specific Tc of control cells expressing the *neo*^{r} gene alone (P4). However after closer examination it appears that this control cell line P4, which has been designated as the neomycin resistance alone control, was in fact produced by co-transfection of a Mo-*env* expression vector with a neomycin resistance vector, but was then assumed negative for *env* expression due to lack of detection of gp70. Use of this cell line as the neomycin resistance alone control now seems inadvisable. It has been shown by other groups that levels of expression undetectable by standard immunodetection methods can be sufficient to effect Tc recognition (e.g. Townsend *et al.* 1984) and furthermore, the *env* protein p15E is now thought to have an immunosuppressive effect. It is thus possible that this cell line is not representative of cells transfected with the *neo*^{r} gene alone.

B) Examination of the *in vivo* growth of *ras* transformed tumour cells expressing specific MLV antigens

i) Analysis of the *in vivo* growth of tumour cells expressing Kirsten specific MLV antigens

The growth of the tumour cell lines MK-gag1, MK-gag2 and MK-*env* in C3H/He mice was examined and the results are shown in table 4.6B.1. It appears from these results (as suggested from the Tc studies) that the MC3H201

Table 4.6B.I. Examination of the in vivo growth of the tumour cell lines NK-gag1, NK-gag2 and NK-env

Tumours scored (/16)

cell line	days post inoculation 7	10	14	18	21
C3H2D1	0	16	16	16 M	16
NC3H2D1	0	0	10	12 S	16
NK-gag2	0	16	16	16 M	16
NK-gag1	0	0	6	9 S	9
NK-env	0	16	16	16 L	16

The mice were inoculated with 3×10^6 cells subcutaneously as described in section 2.13. Tumours were scored by palpation from day 7 post inoculation of tumour cells. At day 18 post inoculation a record of the relative tumour size is also included, with the tumours scored for C3H2D1 designated as medium (S = small, M = medium and L = large). Mice were examined for at least 4 weeks post inoculation of tumour cells and no tumour regression was observed

cells do elicit an immune response; the tumours produced from these cells were smaller and were detectable later than for the control cell line C3H201. Clearly this response to the MC3H201 cells complicates the interpretation of the results from this study. Nevertheless, it does appear that growth of the MK-gag1 cell line is greatly reduced in comparison with both the C3H201 and MC3H201 cell lines. It is therefore suggested that an immune response is being detected against these cells. Similarly, by comparison of MK-gag1 and MK-env with the control cell lines it appears that no response is detected against these cells.

ii) Analysis of the *in vivo* growth of tumour cells expressing Moloney specific MLV antigens

The growth of the tumour cells MK-gag and MK-env in C3H/He mice was examined and the results are shown in table 4.6B.ii. In parallel to the situation described above it appears that these MC3H201 cells induce an immune response; again the tumours (in comparison with the C3H201 tumours) are smaller in size and are not detectable until later post inoculation. Furthermore, fewer tumours in total were scored even after 4 weeks post inoculation. The MK-gag cells show reduced growth in comparison to MC3H201 and C3H201, however the response to the MC3H201 cells makes it impossible to determine the significance. Similar to the MK-env cells above, the MK-env cell line shows no notable reduction in growth.

4.7 Discussion

Many groups have designed retroviral vectors that express two exogenous genes. These vectors are mostly designed in one of the following three ways. Firstly, one gene is transcribed from the LTR and the other gene is transcribed from an internal promotor (e.g. Lu *et al*, 1989; Bowtell *et al.*, 1988). Secondly, both genes are transcribed from the LTR and one of the

Table 4.6B.II. Examination of the in vivo growth of the tumour cell lines NM-gag and NM-env

Tumours scored (/16)

cell line	days post inoculation 7	10	14	18	21
C3H301	0	12	15	15 M	15
NC3H301	0	0	9	12 S	12
NM-gag	0	0	8	10 S	10
NM-env	0	9	15	15 M	15

The mice were inoculated with 3×10^5 cells subcutaneously as described in section 2.12. Tumours were scored by palpation from day 7 post inoculation of tumour cells. At day 18 post inoculation a record of the relative tumour size in mice included, with the tumours scored for C3H301 designated as medium (S = small, M = medium and L = large). Mice were examined for at least 4 weeks post inoculation of tumour cells and no tumour regression was observed.

genes is translated from a spliced transcript (e.g. Rowtell *et al.*, 1988). Thirdly, one gene is transcribed from the LTR from a spliced transcript and the other is transcribed from an internal promotor (Osborne and Miller, 1988). In the selection vectors described in this chapter, transcription of the MLV gene (*gag* or *env*) and the *neo*^{r} gene is driven by an LTR. Both the *gag* and *env* genes are transcribed from the 5' LTR, but the *env* gene products are translated from a spliced transcript. The *neo*^{r} gene in all three vectors is transcribed from the LTR 3' to the MLV gene.

Although many groups have reported the successful use of retroviral vectors to express exogenous genes, problems have been reported in a few cases. Emerman and Temin (1984) designed retroviral vectors that expressed the *neo*^{r} gene and the herpes simplex virus thymidine kinase gene (*tk*) from separate promotors. They found that when a single provirus was integrated into the cell genome and they selected for expression of the 5' gene then most cells in a cell clone were killed when selection pressure for the expression the 3' gene was applied. It was proposed that when selecting for expression of one gene in the integrated provirus the other gene was usually entirely or partially inactivated relative to the amount of expression necessary for growth in the selective medium (Emerman and Temin, 1984).

Later studies into this suppression showed that suppressed genes produced about 10 to 50% as much product as when they were selected and that the supppression acted by altering the amount of RNA transcribed from each promotor (Emerman and Temin, 1986a). When the selected cell lines were cloned, it was found that the amount of suppression varied from clone-to-clone and thus in the bulk population there existed subpopulations expressing different amounts of suppressed gene. Similarly, the results

presented in this section suggest the existence of subpopulations within the NH-gag cell line that express the *gag* gene to varying degrees.

The cell lines used in the studies described above contained only a single copy of the retroviral vector, whereas transfected cell lines, similar to NH-gag, have been shown by other groups to contain multiple copies of the transfected DNA. It was proposed by Emerman and Temin (1986a) that this difference explains the discrepancy between their results and those of others reporting coordinate expression of genes with their own promotors linked to selectable markers in transfected cells (e.g. Girli *et al.*, 1983; Rogiaski *et al.*, 1983). They suggest that the inhibition of a promotor in one gene may be compensated by the many copies of the gene in the cell. Furthermore, they report that when cells that contain a provirus with one inactivated gene are reinfected to introduce a second copy of the provirus, the cells act phenotypically as if there was no inhibition of expression.

It is clear from the literature that cell lines vary in their susceptibility to transfection (Chen and Okayama, 1987). It is also possible that when a resistant cell line is transfected, some of the successfully transfected cells may receive fewer copies of the transfected DNA and in some cases only one. It has been shown by others that a C3H10T½ derived cell line has a low transfection efficiency in comparison to a range of other cell lines (Chen and Okayama, 1987). Thus it is possible that some of the cells transfected with pMNO Mo gag which are also derived from C3H10T½ may have only received one copy of the vector. Thus the variable expression of the *gag* gene observed in the clones of NH-gag could result from suppression similar to that described by Emerman and Temin (1984, 1986a).

Cullen *et al.* (1984) also described suppression that they attribute to inhibition of proximal promotors. They found that early after transfection the 3' LTR of an avian retroviral DNA was prevented from transcribing a downstream gene when the 5' LTR was transcriptionally active. Monathalens, Emerman and Temin (1986) concluded from their work with MLV derived retroviral vectors containing a range of internal promotors that this 'transcriptional interference' described by Cullen *et al.* (1984) could not fully account for the suppression observed by them. For instance, they found that the amount of suppression was independant of the relative strength of the promotors in the vector and the distance between the promotors. However, it is suggested by Emerman and Temin (1986b) that transcriptional interference of the LTR transcript through the internal promotor may explain why the 3' gene is always more suppressed when the 5' gene is selected than the 5' gene is suppressed when the 3' gene is selected.

In the case of the present study the vectors are designed with the selectable gene 3' to the MLV gene. According to the results of Emerman and Temin (1986b) this is the most favourable order for expression of the MLV gene. Additionally, Emerman and Temin (1986b) also reported that promotors in vectors that contain the MLV U3 region (either in the LTR or as the internal promotor) are less suppressed by selection for expression of the other promotor than spleen necrosis virus based vectors with an internal *tk* promotor, as used in their earlier studies (e.g. Emerman and Temin, 1984). However, this suppression may still occur.

From the evidence presented in this chapter it is proposed that the expression vectors described here are functionally active. There is clear evidence at the transcriptional level, mRNA specific for the transfected

gene of interest has been detected for all selected cells, however protein analysis of these cells has proved less conclusive as discussed in detail in section 4.5. To be sure that the correct coding sequence of these proteins are present in the expression vectors it was decided to express the genes *in vitro*. From the preliminary investigations described in this chapter it appears that *gag* is the main T cell target; the *gag* expressing cells are more efficiently killed and are also less tumourigenic than the *env* transfected cell lines. For this reason the *gag* expression vector pUC Mo gag was expressed *in vitro* as described in the following chapter. The *env* coding sequences were not expressed *in vitro* due to insufficient time.

As discussed earlier it appears from the results presented above that the use of a neomycin selection system described in this chapter is inappropriate for the investigations of this study. It can be seen that the cells expressing the neo^R gene alone show increased susceptibility to Tc lysis and are less tumourigenic than the C3H201 cells (the Ki BC3H201 tumours were smaller and were not detectable until a later date and the Mo BC3H201 tumours were also not detected in all the inoculated mice). Neomycin resistance is an ideal selection system under certain circumstances as illustrated by its wide-spread use. However its suitability for *in vivo* studies has clearly been brought into question in this study. In chapter 6 the design, construction and the application of an alternative system utilising the MHC D^b antigen is described.

Nonetheless, it does appear from the Tc killing assays and the tumour growth studies that *gag* is a target in tumour cell recognition *in vitro* and *in vivo*. Furthermore it was found that IFN-γ treatment of the *gag* expressing cells resulted in an increase in susceptibility to Tc killing above that observed for BC3H201. As described before it is proposed that

this increase is due to an increase in class I MHC antigen expression on the cells after IFN-γ treatment. Due to the interference of the neo^R gene in these studies it is difficult to determine the significance of this recognition. It is proposed that the D^b selection system will overcome this problem.

Chapter 5 *In vitro* transcription and translation of the HLV coding sequences of the expression vector pUC Ho gag

5.1 Introduction

Although it has been shown that transcription of the transfected expression vectors does occur, attempts to detect the translated products have been inconclusive due to an inefficient detection system. As discussed at the end of chapter 4, a decision was made to express the products of the subgenomic fragment LTR-gag-LTR (excised from pUC Ho *gag*) *in vitro* and then to examine them by radioimmunoprecipitation (section 2.11C) in order to ascertain whether the Ho *gag* polypeptides are expressed. The *in vitro* transcription and translation systems used were the Riboprobe Gemini™ transcription system and a New England Nuclear rabbit reticulocyte lysate translation kit, respectively. The *in vitro* transcription vectors available as part of the Gemini system contain both the SP6 and T7 promotors separated by a multiple cloning site. In this work the vector pGEM-1 was used as this allowed transcription of the inserted sequences of interest to be achieved using the cheaper of the two enzyme, T7 RNA polymerase.

5.2 Insertion of the Ho *gag* gene into the *in vitro* transcription vector pGEM-1

The construction of the *in vitro* expression vector pGEM-gag is shown in Figure 5.2a. and the methods used are described in section 2.8. A control vector was also produced in which the LTR and *gag* LTR containing fragment was inserted in the wrong orientation for translation from these sequences to occur and is referred to as pGEM-gag. There are no endogenous start codons present in the multiple cloning site of pGEM-1, thus when the *gag* containing fragment is inserted into this vector translation can only occur from the *gag* start codon(s). The pGEM-gag and pGEM-gag plasmid DNAs were digested with a range of restriction enzymes and the results from this

Legend to figure 6.2a. Construction of the pGEN1 *in vitro* expression vector pGEN Mo *gag*. pGEN1 was digested with EcoRI and Hind III, followed by dephosphorylation with CIAP. pUC Mo *gag* was digested with EcoRI and Hind III. The LTR *gag* EcoRI/EcoRI fragment (~3100 bp) and the LTR EcoRI/Hind III fragment (~700 bp) were isolated. The above two fragments were cloned into the EcoRI/Hind III site of pGEN1 via a three way ligation.

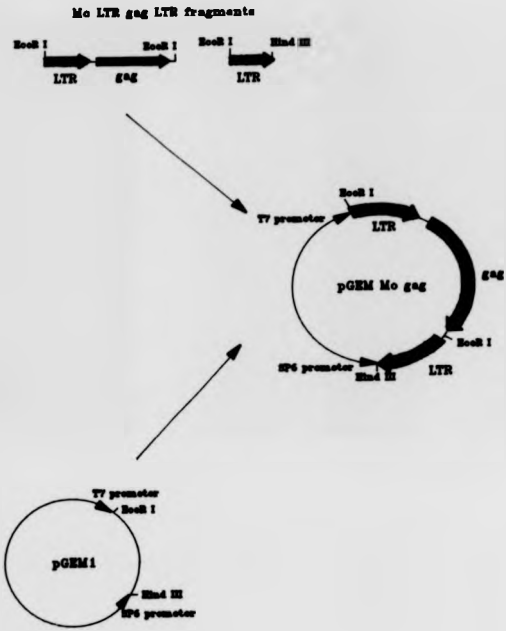
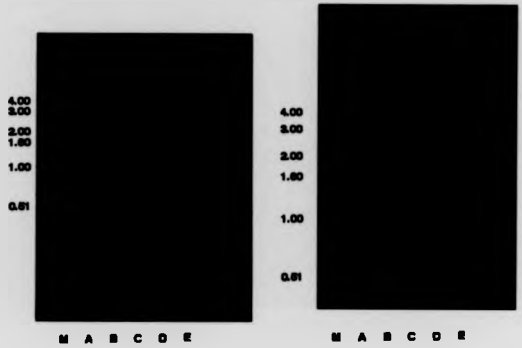


Figure 5.2a. Construction of the pGEM1 in vitro expression vector pGEM Mo gag.



Legend to figure 5.2b. Restriction map analysis of the pGEM1 *in vitro* expression vector pGEM Hu gag. Plasmid DNA was restricted with Hind III/EcoR I (a), EcoR I (b), Hind III (c), Bgl II (d), Xba I (e) and H/A (f). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with Xba I would result in fragments of 2.80, 3.10 and 0.80 kbp.

analysis for pGEM-gag are shown in Figure 5.2b. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data.

5.3 *In vitro* transcription and translation of the Mo *gag* gene

The template DNA was linearised with Hind III and *in vitro* transcription was performed as described in section 2.10C. The RNA produced during a reaction was resuspended in 20 µl of translation grade distilled water and 2 µl of this was used per *in vitro* translation reaction. *In vitro* translation was performed as described in section 2.11I with ^{35}S-Methionine incorporation used to label the protein products. *In vitro* transcription and translation of pGEM-gag and PGEM-gag was carried out concurrently.

5.4 *Analysis of the in vitro* translated products of pGEM-gag and pGEM-gag

At the end of the translation reaction each sample was divided into two, one half was immunoprecipitated with anti-Mo-gag antibody (2.11C) and then both halves were analysed by SDS/PAGE (2.11D). Figure 5.4 illustrates the results of this analysis. It can be seen from lanes A and B that no proteins are translated from the RNA transcribed from pGEM-gag as the only bands in these tracks are due to proteins endogenous to the reticulocyte lysate translation system used. In track C there are several bands in addition to the bands of the endogenous proteins with the major products having the estimated molecular weights of 68 and 72 kD. Two of these bands are also present in track D showing that the proteins they represent are immunoprecipitated by the anti-Mo-gag antibody.

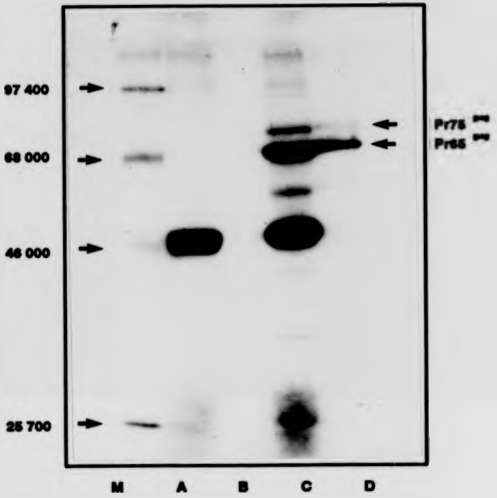


Figure 5.4. Analysis of the *in vitro* translated products of pGEMgag. RNA transcribed from pGEMgag and pGEM$_{gag}$ was translated in a rabbit reticulocyte lysate system containing ^{35}S-methionine. Prior to separation by SDS-PAGE each sample was divided and half was immunoprecipitated with anti-Mo-gag serum.

Track M: molecular weight markers (Daltons)
A: total products of pGEM$_{gag}$ *in vitro* translation
B: immunoprecipitated products of pGEM$_{gag}$
C: total products of pGEMgag *in vitro* translation
D: immunoprecipitated products of pGEMgag

The positions of $Pr65^{gag}$ and $P75^{gag}$ are indicated.

5.5 Discussion

These results concur with the observations made by Edwards and Fan (1979; 1980). They report that when Mo-MLV virion RNA are translated in a rabbit reticulocyte lysate system, a variety of polypeptides are synthesized which are immunoprecipitated with a anti-Mo-gag antibody. The major translation products had molecular weights of 68 and 75 kD in agreement with the observations of section 5.4. It was found that these polypeptides comigrated with *in vivo* labelled $Pr65^{gag}$ and $P75^{gag}$ (the unglycosylated form of $GpP80^{gag}$), respectively and it is proposed that they are in fact the same. The translational products smaller than $Pr65^{gag}$ present in Figure 5.4 track 4 but not immunoprecipitated may represent initiation of protein synthesis from normally internal methionine codons in fragmented RNA. Alternatively the bands which are immunoprecipitated may be the result of premature translation terminations known to occur in the rabbit reticulocyte lysate system. $Pr65^{gag}$, as discussed previously in section 1.4, is the immediate precursor of the internal structural *gag* proteins (p30, p15, p12 and p10) but the function of the glycoprotein $GpP80^{gag}$ (also referred to as $P80^{gag}$) is unclear at present. Pillemer *et al.* (1986) reports that it is intercalated into the membrane by its NH_2 domain and then is further processed to give two glycoproteins of 55 and 45 kD which are released from the cell (Edwards and Fan, 1980). Work by Schwartzberg *et al.* (1983), with viable deletion mutants of Mo-MLV which lack $GpP80^{gag}$, showed that $GpP80^{gag}$ is not required for replication and transmission, however more recently, Prats *et al.* (1989) have reported that this viral protein (renamed $gp85^{gag}$) enhances virus spread early in infection by facilitating virus maturation and infection.

Early investigators proposed that $GpP80^{gag}$ is the primary translation product of the *gag* gene in MLV-infected cells, and that $Pr65^{gag}$ is derived

from it by proteolytic cleavages (Jamjoom *et al.*, 1977; Murphy *et al.*, 1978; Ledbetter *et al.*, 1978). However, Edwards and Fan (1980) showed that the *in vitro* translated putative $Pr65^{gag}$ and $P75^{gag}$ could be labelled with ^{35}S-formylmethionine, which specifically labels the amino terminus, thus suggesting that $Pr65^{gag}$ is not derived from $P75^{gag}$ by cleavage of the amino-terminal portion *in vitro*. In addition, Schultz and Oroszlan (1978) compared *in vivo*-labelled polyproteins from Rauscher MLV-infected cells and reported that $GpP80^{gag}$ differed from $Pr65^{gag}$ only at the amino terminus. These results are in agreement with the work by Edwards and Fan (1980) who showed by partial proteolysis followed by immunoprecipitaiton of cleavage fragments with different *gag*-specific antisera that the additional polypeptide sequences present in $P75^{gag}$ and $GpP80^{gag}$ but absent in $Pr65^{gag}$ are located at or near the amino terminus. Thus, supported by the work of Schwartzberg *et al.* (1983), they propose that $P75^{gag}$ is initiated upstream from the $Pr65^{gag}$ start and then continues in phase into the sequence of $Pr65^{gag}$. However, although three AUG initiator methionine codons are found in the published sequence (Shinnick *et al.*, 1981) upstream of the AUG for $Pr65^{gag}$, none of these codons are in the same translational reading frame as the $Pr65^{gag}$. Thus to explain the synthesis of $P75^{gag}$ it is necessary to examine initiation of translation in more detail.

The scanning mechanism for initiation of translation in eukaryotes was proposed over a decade ago. The scanning model suggests that the 40S ribosomal subunit (carrying $Met\text{-}tRNA_i^{met}$ and various initiation factors) binds initially at the 5'-end of mRNA and then migrates, stopping at the first AUG codon in a favourable context for initiating translation. The most favourable context for initiation is now known to be GCCGCC$\underline{A}$CC$\underline{AUG}$G. However, an initiator codon can usually be designated 'strong' or 'weak' by considering only positions -3 and +4; a purine (usually A) in position -3

followed by a G at +4 is the most important factor affecting translational efficiency. It was originally thought that translation is always initiated from the first AUG codon in a favourable context from the 5' end of the mRNA (as is the case for 90-95% of the 699 vertebrate mRNA sequences examined by Kozak (1987)). More recent studies by Kozak (for review see Kozak, 1989a) show that initiation of translation in eukaryotes is leaky, that is, initiation can occur from further down stream AUGs as well as the first. Furthermore, initiation can occur from non-AUG codons (although with a maximum efficiency of only 5% that of AUG) and the functioning efficiency of such an initiator codon is also dependant on its context as described above (Kozak, 1989b).

These recent observations have enabled Prats *et al.* (1989) to identify possible non-AUG start codons for $Pr75^{env}$. Deletion and site-directed mutagenesis of the sequence upstream of the $Pr65^{env}$ AUG and *in vitro* translation of the RNAs was then used to determine whether synthesis of the $Pr75^{env}$ occured from these initiation codons. They conclude that synthesis appears to be initiated at a CUG codon located within a favourable context (ACCCUGG at position 354). This CUG is in the same reading frame as $Pr65^{env}$ and no termination codon is located between this codon and the $Pr65^{env}$ AUG. Thus it appears that initiation for $Pr75^{env}$ occurs from the CUG at position 354 because it lies in an excellent context, but functions poorly because it is not AUG, thus most 40S subunits advance to and initiate at the start codon for $Pr65^{env}$, a AUG codon in a good context (AATAUGG). Finally, it is proposed that initiation may also occur very inefficiently at a CUG codon located within a less favourable context (GAACUGA at position 327). This may explain why $Pr75^{env}$ made *in vivo* and *in vitro* often appears as a doublet of 75 to 77 kD.

It has already been shown that the LTR is functionally active as a promotor of transcription; e.g. it has been shown to drive transcription of the neomycin resistance gene in the selection vector pNEO (section 4.3), in addition to the evidence from RNA dot-blot analysis showing transcription from the MLV expression vectors (section 4.4). Thus as the subgenomic fragment LTR-*gag*-LTR has now been shown to contain all the necessary information for synthesis of the *gag* proteins $Pr65^{gag}$ and $Pr75^{gag}$, it is suggested that the *in vivo* expression vector pUC Mo *gag* also contains the necessary information for protein synthesis. It is described in the following chapter how cells that express *gag* proteins can be produced without the involvement of neomycin resistance; the latter having been shown previously to interfere with *in vivo* studies.

Chapter 6 The MHC D^b antigen selection system

6.1 Introduction

As described in some detail in chapter 3 it was initially hoped that cells successfully transfected with the MLV genes could be selected by sorting on a flow cytometer, after staining by indirect immunofluorescence. It can be seen from the results presented in chapter 3 however, that the antibodies used to detect either *gag* or *env* proteins are not suitable for this use as they react non-specifically with an antigen on the surface of the *ras* transformed cell line C3H201. It was thus necessary to use an alternative selection strategy. The first to be used was the neomycin resistance selection system as described in chapter 4. This system was chosen as it allowed rapid assessment of the functioning of the expression vectors, however there were reservations concerning the use of the resulting cell lines in *in vivo* studies. It was thought that the bacterial protein itself (an aminoglycoside 3'-phosphotransferase II) may act as a target and thus may affect the immune response to the antigens of interest expressed in these selected cells. Evidence presented in chapter 4 suggests that this is indeed the case. In the present chapter the use of an alternative selection system is described. This system, which is described in some detail in section 1.7, involves the use of the MHC D^b antigen for selection of transfected cells. The H-$2D^b$ gene is transfected into the H-$2D^b$ negative cell line C3H201 (H-2^a) with the DNA of interest and then the cells expressing the D^b antigen are sorted on the FACS following IFN-γ treatment and indirect immunofluorescence staining. Experiments to determine the immunological importance of the antigen of interest are then performed in the F1 progeny from a cross between C3H/He (H-2^k) and C57BL6 (H-2^b) mice which recognise the endogenous and acquired MHC antigens of the transfected cell line as self. Thus it is proposed that this system can be used to

examine the immune response to the proteins of interest without interference from the selection system. It was intended initially that this D^{a} selection system be used in the production of both *gag* and *env* expressing cell lines. However, as yet only a *gag* expression cell line has been produced. The construction and use of this cell line is described in the present chapter. Also described is the production and use of a D^{a} selected cell line that expresses all MLV proteins without producing the virus itself. A comparison of this cell line with the virus producing MoSVC3H cells (which also express the MLV proteins) should facilitate an examination of the effect of virus replication and further subsequent infection on the immune response to MLV antigens.

6.2 Construction of the D^{a} selection vectors

A) The Moloney *gag* expression vector pMo *gag* D^{a}

The construction of pMo gag D^{a} is shown in figure 6.2A.i and the methods used are described in section 2.8. The pMo gag D^{a} plasmid DNA was digested with a range of restriction enzymes and the results from this restriction analysis are shown in figure 6.2A.ii. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. The 10 000 bp D^{a} fragment inserted into pDC Mo gag contains all the transcription and translation factors necessary for expression of this MHC antigen.

B) The Moloney $MLV\psi^{-}$ expression vector pD^{a} $MoLV\psi^{-}$

The construction of pD^{a} $MoLV\psi^{-}$ is shown in figure 6.2B.i and the methods used are described in section 2.8. The pD^{a} $MoLV\psi^{-}$ plasmid DNA was digested with a range of restriction enzymes and the results from this restriction analysis are shown in figure 6.2B.ii. The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector

Legend to figure 6.2A.1. Construction of the MHC D^b antigen selection vector pUC Mo *gag* D^b. The D^b gene was released from pBR327 by digestion with Hind III and the ~10 000 bp fragment was isolated. pUC13 Mo *gag* was digested with Hind III and dephosphorylated with CIAP. The D^b gene was then cloned into this vector via a two way ligation.

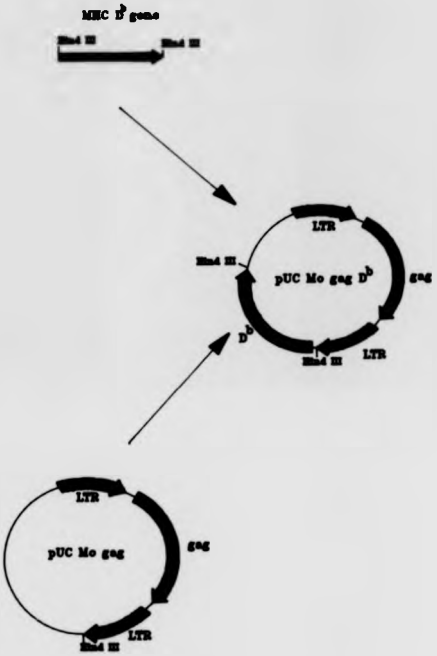
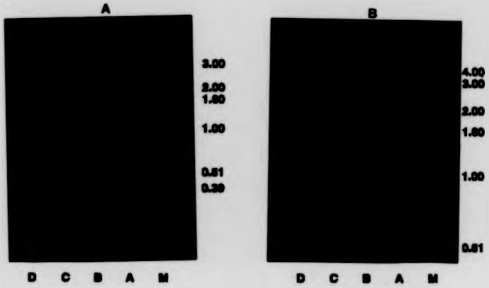


Figure 6.2A.1. Construction of the MHC D^b antigen selection vector pMo gag D^b



Legend to figure 6.2A.11. Restriction map analysis of the MHC H-2D^{p} antigen selection vector pHa gag D^{p}. Plasmid DNA was restricted with Stu I (a), BamH I (b), Hind III (c) and Xba I (d). Restriction fragments were electrophoretically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. For example, restriction endonuclease digestion with BamH I would result in fragments of 0.50, 2.10, 2.50, 4.50 and 6.88 kbp. See Appendix D

Legend to figure 6.2B.1. Construction of the MHC D^b antigen selection vector pD^b Ha MLVγ^-. pBR327 D^b was digested with EcoRI and dephosphorylated with CIAP. pHaVγ^- was digested with EcoRI to release the Ha MLVγ^- fragment. The fragment was isolated and then cloned into the EcoRI site of pBR327 D^b via a two way ligation to form the vector pD^b Ha MLVγ^-.

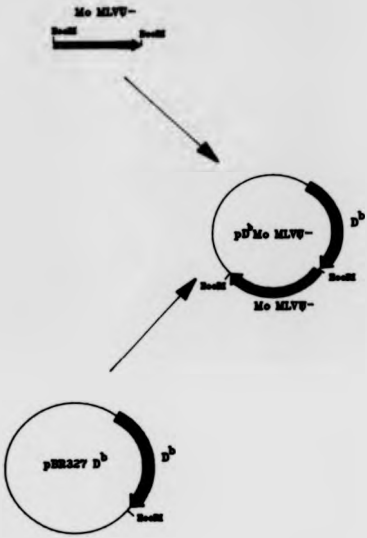
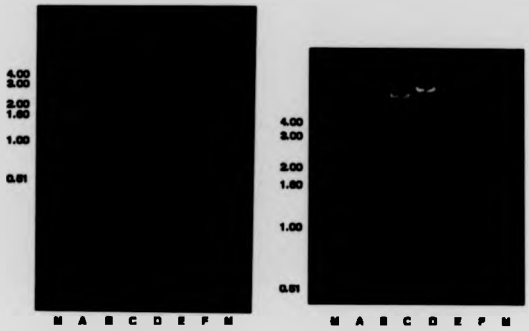


Figure 6.2B.1. Construction of the MHC D^b antigen selection vector pD^b Mo MLVΨ-



Legend to figure 6.2B.ii. Restriction map analysis of the HBC H-2 Dᵖ antigen selection vector pDᵖKo HLVϱ⁻. Plasmid DNA was restricted with EcoR I/Hind III (a), xba I (b), Hind III (c), EcoRI (d), Stu I (e) and H/A (f). Restriction fragments were electrophoratically separated on a 1% agarose gel and visualised with ethidium bromide staining. Figure 1A and Figure 1B represent a short and long run of this gel. The molecular weight markers (given in kbp) are a commercial preparation from BRL (see Addendix C). The patterns of restriction fragments are in agreement with the anticipated restriction map for the vector calculated from the known sequence and restriction map data. See Appendix D.

calculated from the known sequence and restriction map data. The Mo MLVψ⁻ fragment isolated from pMoVψ⁻ is flanked with some mouse cell DNA sequence. Unfortunately due to the absence of an appropriate restriction enzyme site it was not possible to remove this sequence.

6.3 Selection of cells expressing D^b by indirect immunofluorescence and flow cytometry

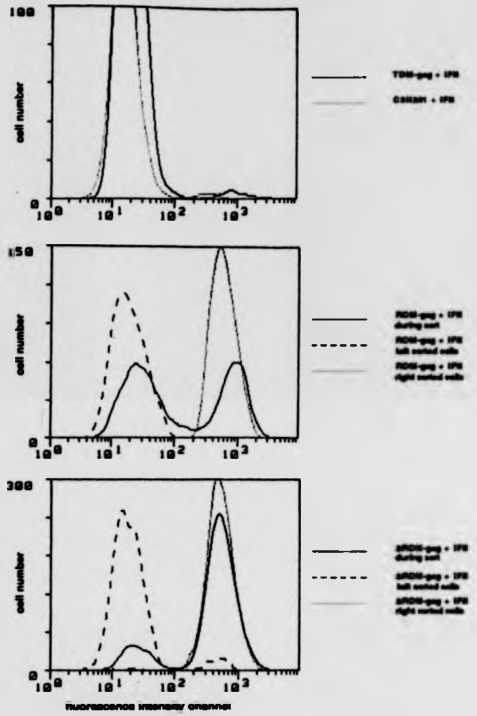
All transfected cells are tabulated in Appendix B.

The expression vectors pMo gag D^b and pD^b MoLVψ⁻ were transfected into the *ras* transformed embryo fibroblast cell line C3H201 either by the method described in section 2.9F or using the Stratagene transfection kit following the manufacturer's instructions. The DNA used for each transfection was of large scale plasmid preparation quality and had been isolated on a caesium chloride gradient. The plasmid DNA of pMo gag D^b and pD^b MoLVψ⁻ were linearised by cutting within the pUC13 and pBR327 sequence respectively, prior to use in a transfection. After transfection the cells were cultured for a further 5 days under normal growth conditions and then were treated with IFN-γ as described in 2.9H to upregulate D^b expression. On day 4 post treatment the cells were examined for D^b expression by indirect immunofluorescence and flow cytometry analysis (section 2.11A). The cell lines G26:24 ($H\text{-}2^a$) and C3H201 ($H\text{-}2^a$) were examined simultaneously with the transfected cell lines as positive and negative controls for D^b staining. A small percentage of the cells transfected with pMo gag D^b were found to express the D^b antigen (~2%), however no such expression was detectable in the pD^b MoLVψ⁻ transfected cell population. The transfection with pD^b MoLVψ⁻ was repeated unsuccessfully several times, thus suggesting either that the vector has difficulty entering the cells or that once in the cells there is a problem with its expression. As this vector is considerably larger than other constructs described in this study it is

possible that the size of this vector may be the cause of the problem (although there was no problem with the construction of this vector in *E. coli* (section 6.2B)). In an attempt to overcome this problem the C3H201 cell line was co-transfected with pMoLVψ⁻ and the D^b gene in a similar manner to that described in section 4.3B. These cells were then examined for D^b expression by indirect immunofluorescence and flow cytometry analysis as described above. Expression of the MHC D^b antigen was detectable in these cells and so these co-transfected cells were used to generate the D^b selected MoLVψ⁻ cell line.

After the flow cytometry analysis described above, the pMo gag D^b transfected and pMoLVψ⁻ & D^b co-transfected cells were then sorted on the same machine; cells expressing the D^b antigen were sorted to the right and non expressing cells were sorted to the left (2.11A). The cells were cultured for 4-5 passages, and then retreated with IFN-γ, restained and resorted. Several cycles of such sorting and culturing resulted in a uniform population of D^b expressing cells for each transfected cell line. After the final sort, the pMo gag D^b transfected and pD^b MoLVψ⁻ & D^b co-transfected cells are referred to as DM-gag1 and DM-MLVψ⁻, respectively (Appendix B). Figure 6.3A shows the indirect immunofluorescence and FACS analysis of the DM-gag1 cells at 3 stages of the sorting procedure. Histogram I shows the cells after transfection (TDM-gag1). The D^b expressing cells from this population were sorted to the right and are referred to as RDM-gag1. The resorting of these cells is illustrated in histogram II and the resulting right sorted cell population is referred to as 2RDM-gag1. Finally histogram III shows the final sort of these cells. The resulting DM-gag1 cell line uniformly expresses the MHC D^b antigen. Thus the sorting procedure employed in this study is successful in selecting cells transfected with the D^b MHC antigen.

Figure 6.3A. Selection of the DM-gag1 cell line by flow cytometry after staining with the anti-H-2D^b antibody by indirect immunofluorescence



8×10^6 cells were stained with the anti-H-2D^b antibody by indirect immunofluorescence as described in the method section 2.11.A. at a dilution of 1/100. Cells were sorted on a FACStar flow cytometer as described in section 2.11.A.

The C3H201 cell line was also transfected with the D^b gene alone and co-transfected with pOC Mo gag and the D^b gene. These cells were sorted in the same way as described above (data not shown) and the resulting selected cells are referred to as DC3H201 and DM-gag2. The DC3H201 cells are used as a control cell line in the following sections.

The cell lines DC3H201, DM-gag1, DM-gag2 and DM-KLVγ^- were all derived from the same C3H201 cells at the same passage number. This is important as it has been shown by other members of the CRC research group that the properties of C3H201 cells vary significantly with passage number post transformation; for instance it has been shown that MHC antigen expression varies with the number of cell generations after transformation (Morris *et al.*, 1989).

6.4 Analysis of mRNA prepared from D^b selected cells

The analysis of mRNA to determine the level of gene expression in DM-gag1, DM-gag2 and DM-KLVγ^- cells was performed by the dot-blot hybridisation procedure, in a similar manner to that described in section 4.4. The mRNA was prepared as described in section 2.10A and was stored before use in a precipitated form in ethanol at -70°C. The DNA probe was prepared from the Pvu I fragment of pZAP (as in section 4.4A.ii) by one of the procedures detailed in section 2.8I.

Figure 6.4 shows the results from this dot-blot hybridisation analysis, with MoSVC3H and DC3H201 as the positive and negative control cell lines, respectively (C3H201 is also shown in this figure). Generally, 5, 10 and 20 µg were dotted onto the nitrocellulose filter for each mRNA sample. Specific hybridisation with the mRNA of MoSVC3H was observed with no detectable non-specific binding with DC3H201 and C3H201. DM-gag2 shows

Figure 6.4. Examination of mRNA from the D^b selected cell lines DM-gag1, DM-gag2 and DM-DLVU-. The mRNA was analysed by dot blot hybridisation as described in section 2.10B. The filter was washed at high stringency (0.1x SSC, 0.1% SDS, 65°C) and the autoradiograph was exposed for 24 hours at -70°C using a Du Pont Cronex intensifying screen. gag-specific probe.

strong specific hybridisation with the radiolabelled probe. DN-gag1 and DN-MLVy⁻ in comparison, show less hybridisation although the level is still clearly well above background. These results indicate the presence of Mo *gag* specific mRNA in all three transfected cell lines; with DN-gag2 expressing the greatest amount.

6.5 Analysis of polypeptides produced by D^b selected cells

The analysis of polypeptides produced by the D^b selected cell lines was performed by either radioimmunoprecipitation followed by one-dimensional SDS-PAGE or SDS-PAGE followed by western blotting (section 2.11). In a similar manner to that described previously (4.5), radiolabelled polypeptides separated by electrophoresis were identified by autoradiography and proteins western blotted onto nitrocellulose were visualised with the appropriate antibody using biotin-streptavidin peroxidase colour labelling. All transfected cell lines are tabulated in Appendix B.

Figure 6.5.1 shows the western immunodetection analysis of polypeptides from DN-gag1 and DN-gag2 with the anti-Mo-gag antibody after separation by SDS-PAGE. The positive and negative control cell lines used were MoSVC3H and DC3H201, respectively. As observed before, it can be seen from track B that this antibody efficiently detects the p30 *gag* protein expressed by MoSVC3H, although no precursor protein $pr65^{gag}$ is detected. Examination of tracks C, D, E and F (cf. negative control track A) indicates that the transfected cell lines DN-gag1 and DN-gag2 express the precursor polypeptide $pr65^{gag}$. Furthermore, it appears that DN-gag2 expresses approximately twice the amount of protein in comparison with DN-gag1, which is in agreement with the results of mRNA analysis described above. In contrast, western immunodetection analysis (with anti-Mo-gag antibody) of

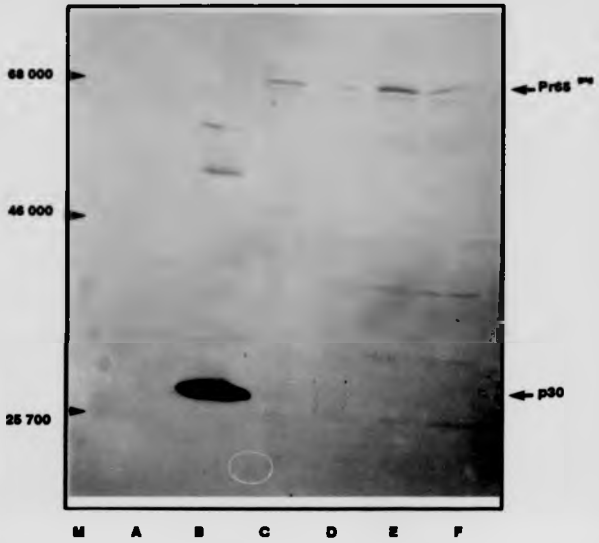


Figure 6.5.1. Examination of the polypeptides produced by the D^b selected cell lines DM-gag1 and DM-gag2. Samples were separated by SDS-PAGE, western blotted and Mo *gag* polypeptides visualised with peroxidase labelled anti-Mo-gag serum.

Track M: molecular weight markers (Daltons)
A: C3H201 (40 µl cell lysate)
B: MoSVC3H (40 µl cell lysate)
C: DM-gag1 (40 µl cell lysate)
D: DM-gag1 (20 µl cell lysate)
E: DM-gag2 (40 µl cell lysate)
F: DM-gag2 (20 µl cell lysate)

The positions of the p30 and $pr65^{gag}$ proteins are indicated.

lysate prepared from DM-MLVψ⁻ cells failed to detect any *gag* specific protein (data not shown). Similarly, radioimmunoprecipitation of the DM-MLVψ⁻ lysate with the anti-Mo-gag antibody, as shown in figure 6.5.ii, also failed to detect any *gag* specific polypeptides. These results suggest that either the DM-MLVψ⁻ cell line does not express any *gag* specific proteins or they are expressed at a level that is undetectable by both western immunodetection and radioimmunoprecipitation. However the mRNA analysis of these cells (discussed in section 6.4) suggests that *gag* specific proteins should expressed although at a significantly lower level than found in MoSVC3H cells.

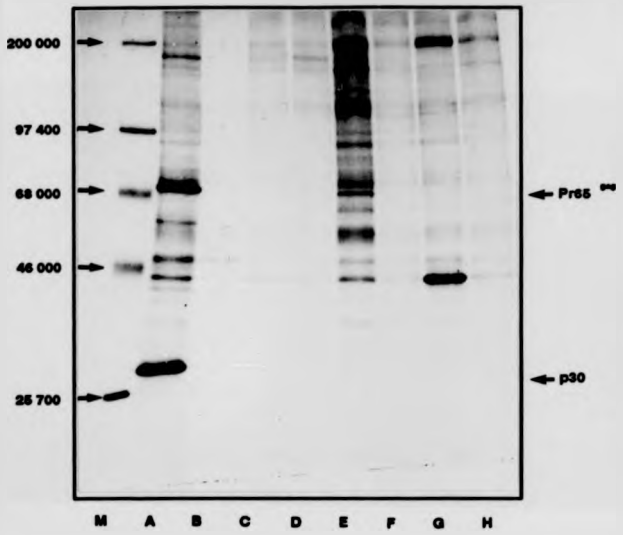


Figure 6.8.ii. Examination of the polypeptides produced by the D^{3} selected cell line DM-MLVU-. Cells were labelled with ^{35}S-methionine and the labelled proteins were immunoprecipitated with the anti-Mo-gag serum and analysed by SDS-PAGE and fluorography using a pre-flashed x-ray film.

Track M: molecular weight markers (Daltons)
A: MoSVC3H
B: DC3H201
C: DM-MLVB- 10 days post second sort
D: DM-MLVB- 20 days post second sort
E: DM-MLVB- 30 days post second sort
F: DM-MLVB- 10 days post final sort
G: DM-MLVB- 20 days post final sort
H: DM-MLVB- 30 days post final sort

The positions of the p30 and $pr65^{gag}$ proteins are indicated.

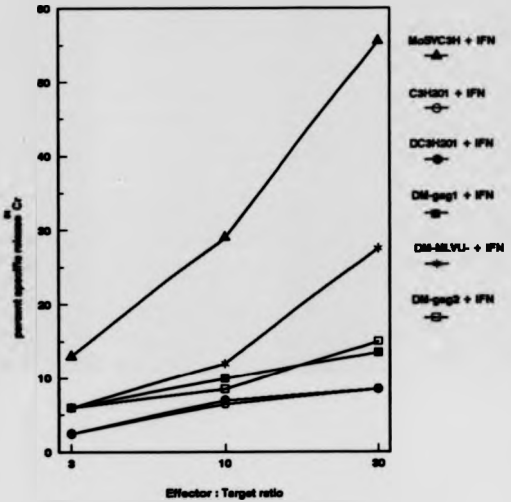
6.6 The use of D^b selected cells to investigate the immune response to specific antigens on tumour cells

In a similar manner to that described in section 4.6 the immune response to these D^b selected cells was examined in two ways. The Tc response to these cells was examined in an *in vitro* chromium release assay (section 2.12B) and the *in vivo* growth of these D^b selected cells in naive and immunized F1 hybrid mice (C3H/He x C57BL6) was observed (section 2.12C). As discussed before the cells used for each study were of approximately the same passage number. All transfected cell lines are tabulated in Appendix B.

A) *In vitro* examination of the Tc response to specific antigens on tumour cells

Figure 6.6A.1 shows the susceptibility of the cell lines DM-gag1, DM-gag2 and DM-MLVγ^- to lysis by Mo-MSV/MLV-specific Tc (all cell lines were IFN-γ treated). It has previously been shown that the procedure used in this study to generate MSV/MLV-specific Tc produces effector populations with no significant levels of natural killer cell activity (section 4.6A). It can be seen here that the MoSVC3H cell line is highly susceptible to lysis by these Mo-MSV/MLV-specific Tc whereas the C3H201 and DC3H201 cell lines were not killed to a significant level. This is in contrast to the results observed for the neomycin resistant cell line NC3H201 which showed increased susceptibility to lysis over the C3H201 cell line (see section 4.7). These results thus appear to support the suitability of this D^b selection system for *such in vitro* studies. It can be seen that all three cell lines DM-gag1, DM-gag2 and DM-MLVγ^- are susceptible to lysis, however the former two are only a third as susceptible as the DM-MLVγ^- cells. These results suggest that all these cells express antigens that are recognised by Mo-MSV/MLV-specific Tc, even though in the case of the DM-MLVγ^- cells no protein could be detected by western immunodetection or radio-

Figure 6.6A.i. The susceptibility of Mo gag and pMoVU- expressing tumour cells to lysis by Mo-MSV/MLV-specific cytotoxic T lymphocytes



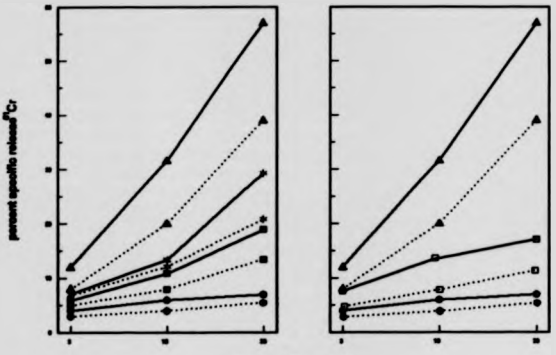
The susceptibility of the cells to lysis by Mo-MSV/MLV-specific cytotoxic T lymphocytes was determined as described in section 2.12.5. These results are the average of 5 replicates from the same experiment (SE ≤ 1.1 % lysis). This experiment was repeated three times and these results are representative.

immunoprecipitation. These results further support the proposal presented in section 4.6A.ii that levels of expression undetectable by standard immunodetection methods can be sufficient for Tc recognition (Townsend *et al.*, 1984).

As discussed before, in contrast to the virus-producing MoSVC3H cell line, DM-MLVγ^- cells were expected to express all the MLV antigens without producing the virus itself. It was thought therefore that these two cell lines could be used to examine the effect of virus replication and further subsequent infection on the immune response to MLV antigens. However, due to the difference in the level of expression of MLV antigens between these cell lines (as shown for *gag* expression, although other antigens were examined) it is not possible to determine any effects due to viral replication. Finally, from examination of the results for the DM-gag1 and DM-gag2 cell lines it is proposed that the *gag* peptide fragments are recognised by the Mo-MSV/MLV-specific Tc generated in this study. This is in agreement with the observations made for the MM-gag1 cell line as discussed in section 4.6A.ii.

The effect of recombinant IFN-γ treatment on the susceptibility of the cell lines DM-MLVγ^-, DM-gag1 and DM-gag2 to lysis by Mo-MSV/MLV-specific Tc is shown in figure 6.6A.ii. In agreement with the results presented in section 4.6A.ii IFN-γ treatment of the MoSVC3H cells appears to increase the susceptibility of these cells to lysis by Mo-MSV/MLV-specific Tc. The DC3H201 negative control cells (in contrast to the MC3H201 cells of figure 4.6A.ii) show no significant increase to lysis by Mo-MSV/MLV-specific Tc, thus further supporting the suitability of this novel selection system. It appears that all three cell lines DM-gag1, DM-gag2 and DM-MLVγ^- display increased susceptibility to Mo-MSV/MLV-specific lysis after IFN-γ

Figure 6.6A.8. The effect of IFN on the susceptibility of the cell lines DM-gag1, DM-gag2 and DM-MLVU- to lysis by Mo-MSV/MLV-specific cytotoxic T lymphocytes



MoMSVC3H + IFN
MoMSVC3H untreated
DC3H201 + IFN
DC3H201 untreated
DM-gag2 + IFN
DM-gag2 untreated
DM-MLVU- + IFN
DM-MLVU- untreated
DM-gag1 + IFN
DM-gag1 untreated

The susceptibility of the cells to lysis by Mo-MSV/MLV-specific cytotoxic T lymphocytes was determined as described in section 2.12.5. These results are the average of 6 replicates from the same experiment (SE ≤ 1.6 % lysis). This experiment was repeated twice and these results are representative.

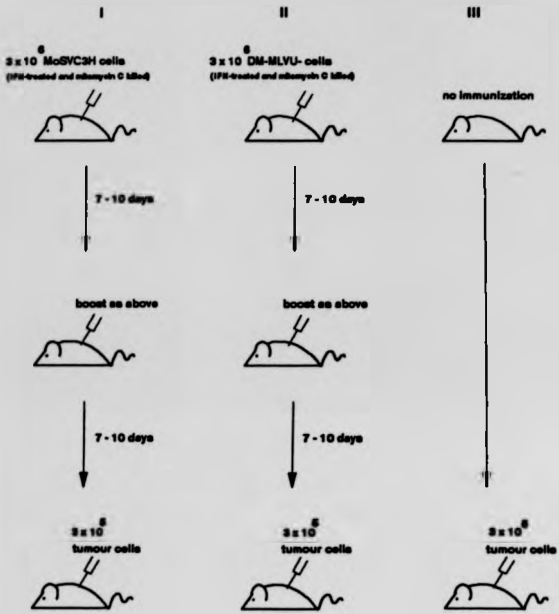
treatment. Furthermore, it appears that the increase in susceptibility observed for DM-MLVγ⁻ is twice that observed for DM-gag1 and DM-gag2. As discussed below, it is proposed that this increase in killing (as observed for MoSVC3H, DM-gag1, DM-gag2 and DM-MLVγ⁻) is due to an increase in class I MHC antigen expression.

B) Examination of the *in vivo* growth of tumour cells expressing specific antigens

The growth of the tumour cell lines DM-gag1, DM-gag2 and DM-MLVγ⁻ in the F1 hybrid mice (C3H/He x C57BL6 cross) was compared in naïve mice, mice immunized with MoSVC3H and mice immunized with DM-MLVγ⁻. The experimental details for this study (including the immunization protocol) are shown in figure 6.6B.1. The results are presented in table 6.6B.1.

It can be seen from a comparison of tables I, II and III that the significant growth observed for DM-gag2 and DM-MLVγ⁻ cells in naïve mice is completely absent in mice immunized with MoSVC3H or DM-MLVγ⁻. χ^2 testing shows this difference to be significant with a 95% confidence level. In contrast, the growth of the DC3H201 cells in naïve mice and the immunized mice are not significantly different. It is suggested from these results that both cell lines DM-gag2 and DM-MLVγ⁻ express antigens that are recognised by the immune system and furthermore the DM-MLVγ⁻ cells appear to immunize mice in a similar manner to the MoSVC3H cells. The results observed with the DM-gag1 cells appear inconsistent with those for DM-gag2. The two cell lines, although derived from different transfections, were found by RNA and protein analysis to have similar levels of *gag* expression (see above). Furthermore, they were found to be equally susceptible to lysis by Mo-MSV/MLV-specific Tc and therefore were expected to exhibit similar tumour growth patterns. This appears not be the case as DM-gag1

Figure 6.6B.I. Immunization protocol for the tumour growth study shown opposite.



Results of the tumour growth study are shown in Table 6.6B.I opposite.

Table 6.6B.I. Examination of the in vivo growth of the tumour cell lines DM-gag1, DM-gag2 and DM-MLVU-

Tumours scored (/10)

	cell line	days post inoculation 7	10	14	18	21
I	DC3H301	0	7	9	9 M	9
	DM-gag1	0	0	0	0	0
	DM-gag2	0	0	0	0	0
	DM-MLVU-	0	0	0	0	0

	cell line	days post inoculation 7	10	14	18	21
II	DC3H301	0	8	9	9 M	9
	DM-gag1	0	0	0	0	0
	DM-gag2	0	0	0	0	0
	DM-MLVU-	0	0	0	0	0

	cell line	days post inoculation 7	10	14	18	21
III	DC3H301	0	8	10	10 M	10
	DM-gag1	0	0	0	0	0
	DM-gag2	0	0	8	7 S	7
	DM-MLVU-	0	0	8	8 S	8

The immunization protocol for this data is shown in Figure 6.6B.I. opposite. Tumours were scored by palpation from day 7 post inoculation of tumour cells. At day 18 post inoculation a record of the relative tumour size is also included, with the tumours scored for C3H301 designated as medium (S = small, M = medium and L = large). mice were examined for at least 4 weeks post inoculation of tumour cells and no tumour regression was observed.

cells (which in fact express less $pr65^{gag}$ than DM-gag2) show no growth in naive mice in contrast to the significant growth observed for DM-gag2 cells. Further investigations are required to determine the significance of this difference.

6.7 Discussion

From mRNA and protein analysis of the DM-gag1 and DM-gag2 cells it appears that the pMo gag D^b expression vector is functionally active, that is, can be used to generate a $pr65^{gag}$ expressing cell line by D^b selection as described above. In contrast the pD^bMo MLVψ^- expression vector appears to be defective. The Mo MLVψ^- expressing cell line pD^bMo MLVψ^- was generated however by co-transfection of the D^b gene with the pMo MLVψ^- vector although evidence for expression was only found at the mRNA level. All three cell lines DM-MLVψ^-, DM-gag1 and DM-gag2 were found to be susceptible to lysis by Mo-MLV-specific Tc and this lysis was increased by IFN-γ treatment. As discussed before it is now known that Tc usually recognise antigen in association with class I MHC antigen. Furthermore IFN-γ treatment of C3H201 cells has been shown to increase class I MHC antigen expression on these cells. Thus it is proposed that the increased susceptibility to lysis observed for these IFN-γ treated cells is due to increased class I MHC antigen expression. It can be seen that the DM-MLVψ^- cells are approximately twice as susceptible to lysis by the Mo-MSV/MLV-specific Tc as the DM-gag1 and DM-gag2 cells. It has been shown that the DM-gag1 and DM-gag2 cells express significantly more *gag* polypeptide than DM-MLVψ^- cells (the latter with only mRNA evidence of expression). It is thus proposed that the DM-MLVψ^- cells are also recognised by Tc specific for other MLV antigens, possibly *env*. Clearly the generation of an *env* expressing cell line using the D^b selection system would clarify this situation. The absence of significant killing of the DC3H201 control cells

shows that the lysis observed for the DN-MLVγ⁻, DN-gag1 and DN-gag2 cells is not due to the D^a antigen. Thus these results support the suitability of this D^a selection system for *in vitro* Tc studies in which the effector population is derived from F1 hybrid mice.

The tumourigenicity of the DN-MLVγ⁻ and DN-gag2 cell lines appears to be greatly reduced in the immunized mice in comparison with the naive mice; and furthermore immunization with either MoSVC3H or DN-MLVγ⁻ appears to be equally successful. This initially seemed very surprising as the MoSVC3H cells have been shown to express high levels of the MLV proteins whereas only mRNA evidence has been presented for the DN-MLVγ⁻ cells and therefore it was thought that the former would be the more efficient at immunizing mice. After further consideration it is now suggested that the number of cells used for the immunizations (see figure 6.6B.1) may have been in great excess of the required amount. It is proposed that if the cell doses were titrated then the lowest cell number that resulted in immunization would be greater for DN-MLVγ⁻ than MoSVC3H. The reduced tumourigenicity observed for DN-MLVγ⁻ and DN-gag2 in the immunized mice suggests that these cell lines express antigens that are recognised by the immune system of the F1 hybrid mice. It is shown by the DC3H201 cells that the D^a MHC antigen is not recognised as foreign. It is thus proposed that that *gag* peptide fragments expressed by the DN-gag2 cells are recognised by the F1 hybrid mice as foreign. Furthermore the DN-MLVγ⁻ cells also express proteins recognised by these mice even though they are not detected by western immunodetection or radioimmunoprecipitation. These results are in agreement with the Tc studies. From both studies it is apparent that the *gag* polypeptide is recognised by the immune system. There appears to be a relationship between the tumourigenicity of DN-MLVγ⁻ and DN-gag2 and their susceptibility to Tc

lysis thus possibly supporting the importance of the Tc element in the immune response to these cells.

Finally, it is proposed that this novel MHC D^b antigen system is a suitable method of selection for cells to be used in immunological studies. Indeed, the evidence presented in chapter 4 suggests that it is more suitable than the more commonly used neomycin resistance selection system. It is hoped that this selection system will be used in other similar studies. As described in the introduction it is already being used in our group to determine the relationship between the point mutations at codon 13, 14, 59 and 61 of the *ras* oncogene and tumourigenicity (as opposed to *in vitro* transformation). Permutations of these point mutations have been created by site directed mutagenesis and expression vectors containing these various *ras* genes and the D^b gene are being constructed. Following the transfection of these vectors into C3H10T½ cells, the positive cells will be selected as described previously and their tumourigenicity will be determined in F1 hybrid mice.

Chapter 7 Conclusions and future work

It appears from the data presented in this thesis that the retroviral expression vectors described here are functionally active. There is clear evidence at the transcriptional level, mRNA specific for the gene of interest (either *gag* or *env*) has been detected in all selected cells, however evidence at the protein level for the NK-gag1, Nk-gag2 and the DM-MLVψ^- cell lines has not been obtained. This is thought to be due to inefficient detection of the precursor protein $pr65^{gag}$ for the NK-gag1 and NK-gag2 cells and a combination of low level expression (cf. MoSVC3H) and an inefficient detection system for the DM-MLVψ^- cells. As there is evidence of expression at the mRNA level and detectable immune responses to these cell lines it is proposed that the pNEO Ki gag and pNo MLVψ^- expression vectors, from which they derived, are functionally active. It can be seen from the mRNA analysis of the NM-gag cell line that within a transfected population there is a variable level of expression. The NM-gag clone A clearly expresses a high level of *gag* proteins, however immunological studies with cloned cell lines have been avoided as C3H201 cloned lines have been shown to display varying degrees of tumourigenicity in mice by other members of the CRC group (data not presented).

From the immunological studies using the neomycin selected cell lines it was found that the *gag* expressing cells were more susceptible to lysis by MSV/MLV-specific Tc and this lysis was increased by IFN-γ treatment, furthermore these cells were also less tumourigenic than the *env* expressing cells. Although this evidence suggests that *gag* is the more important T cell target in this system, the complications observed due to the expression of the neo^R gene alone (increased susceptibility to Tc lysis and reduced tumourigenicity of the NC3H201 cells in comparison to the C3H201

cells), make it difficult to determine the significance of this recognition and bring into question the suitability of the neomycin resistance selection system for use in *in vivo* studies.

The evidence presented in chapter 6 indicates that the MHC D^b antigen selection system developed in this work is a suitable method of selection for cells to be used in immunological studies. It was found that the cells expressing D^b alone (DC3H201) showed no increase in their susceptibility to lysis over the untransfected C3H201 cells (in contrast to the situation with the MC3H201 cells). From the immunological investigations with the D^b selected cells it was found that the *gag* expressing cells DM-gag1 and DM-gag2 and the DM-MLVψ⁻ cells all are susceptible to lysis by Mo-MSV/MLV-specific Tc and this lysis was increased by IFN-γ treatment. It is known that Tc usually recognise antigen in association with class I MHC antigen. Furthermore, IFN-γ treatment of C3H201 cells has been shown to increase class I MHC antigen expression on these cells. Thus it is proposed that the increased susceptibility to lysis observed for these IFN-γ treated cells is due to increased class I MHC antigen expression. These cells also show reduced tumourigenicity in mice in comparison with DC3H201 and C3H201 (data not shown) thus confirming the recognition of *gag* by the immune system (in the case of DM-gag1 and DM-gag2) and furthermore the correlation between the tumourigenicity and susceptibility to Tc lysis of all these cells supports the importance of the Tc element in the immune response to these tumour cells.

The tumour cell lines generated in this work, have been shown to express well-defined antigens (expression only shown at the mRNA level for DM-MLVψ) that have demonstratable immune responses to them. These cells can now be used to dissect further the nature of the immune response to tumour cells

expressing well-defined antigens and particularly to examine the importance of interferon (IFN) modulation of T cell mediated immune responses to these antigens. Unfortunately there is insufficient time to carry out any further investigations as part of this study, however it is hoped that future work extending from this study will be fruitful. To further the preliminary findings concerning the relative importance of *gag* and *env* in MSV/MLV tumour regression it is essential that an *env* expressing cell line is generated using the novel D^b selection system established in this study. Finally, it is hoped that this selection system will be used by other groups when producing cells to be used in immunological studies.

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APPENDIX A

Oligonucleotide A

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          CTAGTTAGCTAACTAG  BamHI
Spe I  AATCGATTGATCCTAG
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Oligonucleotide B

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Sac I  CGTTGACAATGAAAGACCCCTTCATAAGGCTTAGCAAGCTAGCTGCA
   TCGAGCAACTGTTACTTTCTGGGGAAGTATTCCGAATCGTTCGATCG  Pst I
```

APPENDIX B.

Tabulation of the transfected cell lines (all cells C3H201 derived).

Name	Transfected gene(s)	Transfection method *
DC3H201	D^b	-
DM-gag1	D^b & Mo *gag*	l
DM-gag2	D^b & Mo *gag*	c
DM-MLVψ-	D^b & Mo MLVψ-	l
MoLV201	Mo MLV	-
MC3H201	Neo^R	-
MK-gag1	Neo^R & Ki *gag*	l
MK-gag2	Neo^R & Ki *gag*	c
MM-gag	Neo^R & Mo *gag*	l
MK-env	Neo^R & Ki *env*	l
MM-env	Neo^R & Mo *env*	c

* c = co-transfection
  l = ligated

APPENDIX C. DNA ladder

TITLE

A STUDY OF IMMUNE RESPONSES TO *RAS* TRANSFORMED TUMOUR CELLS EXPRESSING WELL-DEFINED ANTIGENS

AUTHOR

Jennifer Linda Johnson

INSTITUTION and DATE

University of Warwick (1991)

CAMERA 2.